Doctoral Dissertation

Inhibitory effect of plant leaf extracts on carbohydrate digestive enzyme: a case study on some woody plants species collected in Bangladesh and Japan

（植物葉抽出液の炭水化物分解酵素の阻害効果：
バングラデシュおよび日本で採取した木本に関する事例研究）

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Contents

Chapter 1
General Introduction

Plants contain various types of active components, some of which have enzyme inhibitory activity. The carbohydrate digestive enzyme, α-glucosidase, is the key enzyme located in the brush border surface membrane of intestinal cells, which catalyzes the final step in the process of carbohydrate digestion. α-Glucosidase inhibitors reduce blood glucose level by delaying the digestion of carbohydrates by the enzyme and can thus be used to control diabetes.

1.1 Diabetes

Diabetes is a common chronic metabolic disease, characterized by high blood sugar level that results in various health complications, including heart disease, kidney disease, retinopathy, and neuropathy. The prevalence of diabetes is increasing worldwide, and higher prevalence rates of this disease have been observed in Western Pacific and Southeast Asia; the number of diabetes patients in 2013 was 138 million in the Western Pacific and 72 million in Southeast Asia (Table 1.1), and the overall diabetes population

Table 1.1 Regional estimates of diabetes rates by 2013 and 2035 (people aged 20 – 79) (IDF, 2013).

Region of IDF	2013		2035		Increase in rate of diabetes (%)
	Population (million)	People with diabetes (million)	Population (million)	People with diabetes (million)	
Africa	407.9	19.8	775.5	41.5	109.6
Europe	658.7	56.3	668.7	68.9	22.4
Middle East and North Africa	374.5	34.6	583.7	67.9	96.2
North America and Caribbean	334.9	36.8	404.5	50.4	37.3
South and Central America	300.5	24.1	394.2	38.5	59.8
Southeast Asia	883.2	72.1	1216.9	123.0	70.6
Western Pacific	1613.2	138.2	1818.2	201.8	46.0
World	**4572.9**	**381.8**	**5861.9**	**591.9**	**55.0**

was 382 million worldwide (Table 1.1). The numbers of patients with diabetes are higher in developed countries than developing countries.

By 2035, the total numbers of people with diabetes have been projected to rise to 201 million in Western Pacific and 123 million in Southeast Asia (Table 1.1), and to 591.9 million worldwide (Table 1.1). Fifty-five percent of the worldwide population with diabetes live in Southeast Asia and Western Pacific region. Bangladesh was selected for this study as it is located in Southeast Asia where the size of the diabetic population is high.

1.2 Prevalence of diabetes in Bangladesh

Figure 1.1 shows the prevalence of diabetes in adults according to age (IDF, 2015). The dotted line indicates the worldwide prevalence of diabetes, while the black and red lines indicate the prevalence rates of diabetes in Southeast Asia and in Bangladesh, respectively. The ratio of diabetes is increasing among people over 50 years old in Bangladesh (IDF, 2015) (Fig. 1.1). One reason for this increase is rapid economic growth. Bangladesh had the second most rapidly growing economy in 2015, with gross domestic product (GDP) of 6.51% (http://www.tradingeconomics.com/bangladesh/gdp-growth). This is accompanied by changes in both diet and lifestyle, and therefore countermeasures against diabetes are required.

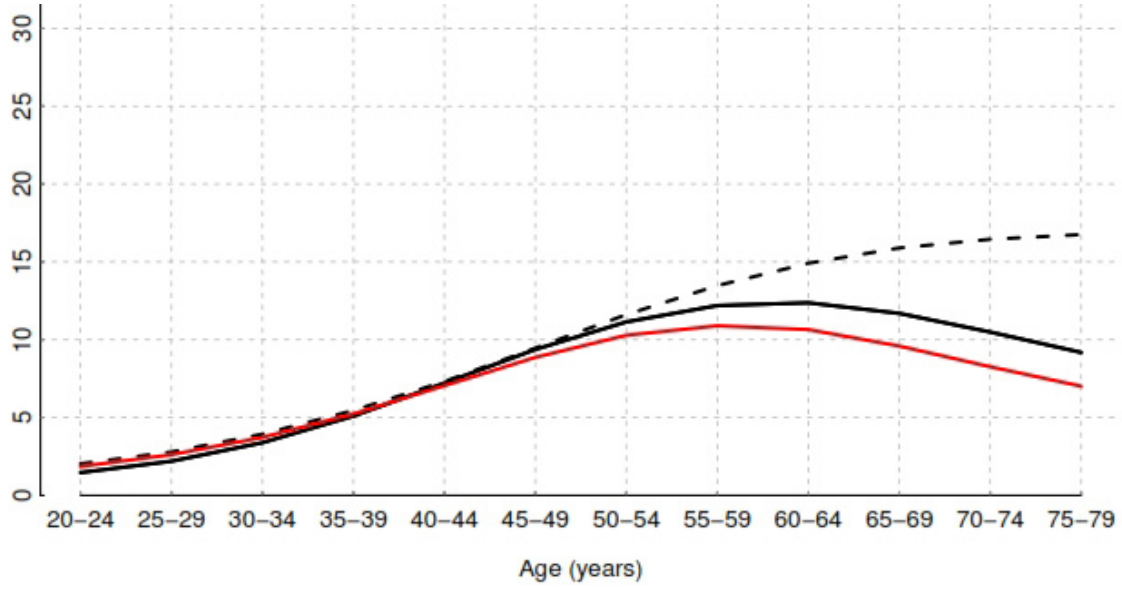

Fig. 1.1 Prevalence of diabetes in adults by age in Bangladesh, 2015

1.3 Medicinal plants in Bangladesh

The use of traditional medicinal plants is very common in Bangladesh, and its geographic location is suitable for growing various medicinal plant species. Table 1.2 shows the 28 most widely used medicinal plant species from the medicinal plant database of Bangladesh (Metabolomics JP: www.metabolomics.jp). These data were collected from local people and traditional practitioners by Prof. Rahmatullah. The different parts of these plants were used to treat the different disease (Table 1.2). Some plant parts were mixed with other plant parts and used to treat a particular disease (Table 1.2).

Table 1.2 Traditional medicinal plants in Bangladesh (Information collected by Prof. Rahmatullah, University of Development Alternative, Bangladesh)

Scientific name	Local name	Parts used	Communicable diseases	Non-communicable diseases
Abelmoschus moschatus	Kalo kasturi	Paste of leaves is applied		Lesions, itches, erectile dysfunction
Abrus precatorius	Sona kuchi	The roots and stems of *Abrus precatorius* are crushed with leaves of *Tinospora cordifolia*, made into a paste, slightly warmed, and ingested.	Tuberculosis	Throat pain
Acacia arabica	Babla	Flower buds of *Hibiscus rosa sinensis*, aerial roots of *Ficus benghalensis*, one clove, and gum from *Acacia arabica* are blended together and ingested with a small amount of ginger juice.		Puerperal fever
Acorus calamus	Boch	The rhizome of young plants is made into a paste, which is then applied externally.	Diarrhea, coughs	Gastrointestinal disorders
Alocasia macrorrhizos	Man kachu	Whole plant		Arsenic antidote
Bambusa multiplex	Tirwa	Ten drops of leaf juice are taken for fever. Leaf and root paste is applied to abscesses.		Fever, abscess, itches
Bryophyllum calycinum	Pathar kuchi	Juice from the leaves is used.		Stomach pains, urination
Cajanus cajan	Arhal	Leaf juice is taken with molasses twice daily for seven days.		Jaundice
Datura metel	Dhutura	Juice of the leaves is used.	Coughs and dysentery	
Emblica officinalis	Amloki	Unripe fruits are chewed for strong teeth; paste of fruits is applied to hair to keep it healthy. Fruits powder of *E. officinalis*, *T. bellirica*, and *T. chebula* are taken in equal proportions and mixed with honey to treat long-term fever and loss of appetite		Teeth and hair problem, long-term fever, loss of appetite
Eclipta alba	Kesharaj	Leaf		Liver disorders
Glinus lotoides	Deema	Leaves and branches washed followed by crushing on a *shil-nora* to extract juice. The juice is filtered and ingested.		Brain cancer, high cholesterol
Gnaphalium luteoalbum	Natham	The plant is crushed along with dried fish and applied as a poultice to heal fractured bones. It has to be kept for 3 – 4 days, frequently wetting with water.		Bone fractures

Table 1.2 (continued)

Scientific name	Local name	Parts used	Communicable diseases	Non-communicable diseases
Ixora parviflora	Hokhu-chutri	Root, stem, and leaf are boiled in water and bath, powder is applied to the affected area	Scabies, leprosy, eczema	
Hibiscus vitifolius	Bon karpas	Juice from the roots		Kidney dysfunction
Hoya parasitica	Dupui-tha	Fruits are either taken with vegetables or dried, powdered, and 2 – 3 pinches of the powder are taken. Note: higher dose will results in diarrhea.		Constipation
Jatropha curcas	Jamalgota	Seed, root	Lesions	Colic pain
Justicia gendarussa	Jagat madan	Leaves are burned and the ashes applied to the affected areas.	Scabies, eczema, allergy	
Lasia spinosa	Joka	The woody root of the plant is eaten daily for 1 week to reduce joint pain and edema. The leaf juice is taken to increase mother's milk.		Joint pain, edema
vvvLeea indica	Chila-khor	Leaf, root		Chronic dysentery
Madhuca indica	Moa	The fruits are boiled with unripe gram and sugar added until the decoction takes on a blood red color.		Debility, blood purifier
Maesa sp.	Allram	Root paste is applied		Urinary problems
Maranta arundinacea	Ararut	Root		Spleen disorder
Ocimum sanctum	Kalo tulshi	Juice from the leaves	Common cold	Malaria
Plumbago indica	Agnichita	Leaf		Ophthalmia
Sida cordifolia	Berela	Leaf juice is taken once every morning for a month as a remedy for nerve weakness. Bark of the root is powdered and one teaspoon of powder taken with 15 – 20 ml of cold water daily.		Nerve weakness, Urinary problems
Terminalia bellirica	Bohera	Inner parts of fruits are taken for diarrhea; dried pulp is taken for cough relief. Fruits of *E. officinalis*, *T. bellirica*, and *T. chebula* are soaked in water and the water drunk every morning for gastrointestinal disorder.	Diarrhea, cough	Gastrointestinal disorder
Terminalia chebula	Haritaki	Paste of fruits and roots is taken twice a day	Cough	Asthma

The medicinal plant database of Bangladesh includes 259 genera, some of which contain only a single species, others contain two species, and some contain three or more species. The different plant species belonging to a given genus may be used to treat the same or different diseases. Therefore, the total number of plant species included in the database is more than 259. In some cases, a similar plant was used to treat various diseases in different villages. The largest number of plant species were used to treat gastrointestinal diseases, with a total of 94 species used for these purposes (Table 1.3). Respiratory problems were treated with 65 species, skin diseases with 63 species, urinary

Table 1.3 Numbers of Bangladeshi traditional medicinal plant species used to treat different diseases.

Name of disease	Number of plant species used for treatment
Gastrointestinal problems	94
Respiratory problems	65
Skin diseases	63
Urinary problems	35
Sexual diseases	31
Fever	28
Liver diseases	22
Diabetes	18
Others	27
Gastrointestinal problems	94

problems with 35 species, sexual diseases with 31 species, fever with 28 species, liver disease with 22 species, and diabetes with 18 species. Twenty-seven species were used for treating other diseases (edema, body pain, cancer, tumor, eye problems, blood-related diseases, dental problems, ulcers, insomnia, narcotic, menstrual disorders, cardiovascular disease, nervous system disorders, and weakness) (Table 1.3). Although many medicinal plants are using to treat various types of disease in Bangladesh, very few plant species are used to control diabetes in this country (Table 1.3). The most widely used medicinal plants (28 plants) were not used for diabetes, because the number of diabetes patients in Bangladesh is still low (Table 1.2). However, the numbers of patients with diabetes are likely to increase because people 56 – 59 years old are developing diabetes in Bangladesh (Fig. 1.1). Therefore, medicinal plants were used in these experiments.

1.4 Background and purpose

This research had two main aspects: (1) to identify new medicinal plants from Bangladesh for diabetes, and (2) to identify new compounds from Japanese plant leaves for diabetes. Many people use traditional medicine in Bangladesh to treat various diseases. About 80% of rural people are dependent on traditional medicine for their primary healthcare, such as a coughs, colds, fevers, headaches, and dysentery (Hossain, 2005). For the treatment of diabetes, plants have been used in various systems of medicine from ancient times, especially in developing countries where large numbers of people have limited access to natural sources and access to allopathic treatment for diabetes (Ali *et al.*,

2006; Nickavar and Yousefian, 2009). Therefore, three Bangladeshi medicinal plants were screened for antidiabetic effects.

Japanese people use traditional medicine occasionally, which is known as Kampo. In Japan, the amount of the use of traditional medicine is not large as modern medicine. In Japanese hospitals and clinics, modern medicine facilities were dominant although traditional medicine is partially practiced there (Park *et al*., 2012). Therefore, we used 19 types of Japanese woody plant leaves to investigate the inhibitory effects on diabetes and identify the structures of active compounds to make modern medicines.

1.5 Antidiabetic compounds of some plants

The digestive enzyme, α-glucosidase, breaks down carbohydrates, such as starch and disaccharides, to *glucose.* α-Glucosidase inhibitors reduce the rate of digestion of carbohydrates, and the carbohydrate absorption process is delayed resulting in a decrease in blood glucose level.

Oki *et al*. (1999) reported that some natural compounds have been shown to have α-glucosidase activity. Among them, flavonoids are the major class of polyphenolic compounds, which are present in many plants and plant parts and have protective effects against many diseases. Flavonoids are polyphenolic compounds that play major roles in the treatment of diabetes (Babu *et al*., 2013). It has been reported that some natural compounds have efficacy against diabetes (Table 1.4).

Table 1.4 Antidiabetic compounds of some plants

Plant	Plant parts	Compounds	Extract	References
Salacia reticulata	Root, stem	Sulfated kotalanol, Ponkolanol, Salaprinol	Water	Muraoka *et al.*, 2008
Belamcanda chinensis	Leaves	Daidzin and Genistein	Water	Wu *et al*., 2012
Ocimum sanctum	Leaves	Polyphenols, caffeic acid	Water	Wongsa *et al*., 2012
Stevia rebaudiana	Leaves	Alkaloids, flavonoids	Water	Kujur *et al*., 2010
Curcuma longa	Rhizome	Curcumin, ar-turmerone	Ethanolic	Kuroda *et al*., 2005
Eleutherine americana	Bulb	Eleutherinoside A	Methanolic	Ieyama *et al*., 2011
Fagara tessmannii	Bark	Terpene	Methanolic	Mbaze *et al*., 2007
Psidium guajava	Leaves	Leteolin, Catechin Kaempferol	Water	Alagesan *et al*., 2012
Adhatoda vasica Nees	Leaves	Alkaloid	Methanolic	Gao *et al*., 2008
Matricaria chamomilla	Leaves	Apegenin, luteolin	Water	Kato *et al*., 2008
Rhodiola crenulata	Root	Epicatechin gallate, epicatechin	Water	Chu *et al*., 2014

1.6 Carbohydrates

Carbohydrates in the diet are metabolized to the monosaccharide, glucose, in the human body by enzymatic processes from the mouth to the small intestine. *Uncontrolled* high *blood* glucose level can lead to *diabetes.* Blood glucose level is affected by intake of carbohydrates, the most abundant nutrients in the diet, with an average percentage of 19.2%.

Carbohydrates are classified into three major classes: monosaccharides, disaccharides, and polysaccharides. Glucose, fructose, and galactose are monosaccharides, which are absorbed directly via the small intestine. Sucrose, lactose, and maltose are disaccharides, which are broken down into monosaccharides for absorption in the small intestine by specific enzymatic activity. Starch and fiber are polysaccharides, among which fiber is resistant to the enzymatic action, while starch breaks down in the mouth by enzymatic activity.

1.7 Carbohydrate digestive enzymes

The most *common* and *abundant* carbohydrate ingredients in the diet are sugar and starch. The final step of carbohydrate digestion is the key step in regulating blood glucose level. The carbohydrate digestive enzyme, α-glucosidase (maltase and sucrase) is an enzyme that catalyzes the final step in the process of carbohydrate digestion in the small intestine. Delaying carbohydrate digestion and absorption through the inhibition of α-glucosidase activity is a useful treatment for diabetes. The inhibition of α-glucosidase can delay the carbohydrate digestion process in the small intestine and finally control postprandial hyperglycemia (Van de Laar, 2008). This approach is one of the best strategies for the treatment of diabetes. Therefore, two digestive enzymes, maltase and sucrase, were selected in this study.

In general, carbohydrate (substrate) binds the catalytic site on the enzyme to form the enzyme–substrate complex. The product (glucose) is produced when the enzyme–substrate complexes breaks down and releases the free enzyme. Thereafter, glucose is absorbed via the small intestine and is circulated in the blood. α-Glucosidase inhibitors are needed to control high blood glucose levels. The α-glucosidase inhibitor binds to the active site of the enzyme, thus preventing the carbohydrate substrate binding

to the active site and preventing its breakdown into glucose. Consequently, the α-glucosidase inhibitor decreases hydrolytic cleavage of carbohydrate, and thus overall glucose absorption is reduced and diabetes can be controlled.

Several antidiabetic agents have been reported. Three α-glucosidase inhibitors, acarbose, miglitol, and voglibose, which can delay carbohydrate absorption in the small intestine and reduce the blood sugar level, are widely used as oral antidiabetic drugs (Tiwari and Rao, 2002). However, these drugs have frequent gastrointestinal side effects, such as flatulence, diarrhea, and stomachache (Van de laar, 2005). Therefore, there is interest in screening of natural products isolated from different plant extracts to find new natural antidiabetic agents with less side effects.

1.8 Targeted plants

This study focused on three plants, *Emblica officinalis*, *Terminalia bellirica*, and *Terminalia chebula*, which have been used for thousands of years in traditional Ayurvedic medicine to treat various types of disease in Bangladesh and India. However, the leaves of these three plants have not been used in traditional medicine. Therefore, this study examined the medicinal effects of the leaves of these three Bangladeshi medicinal plants (Chapter 2). These three plants are used in various ways in traditional medicine in Bangladesh. Pastes made from the bark and roots are applied to affected areas of the skin. Powder prepared from the fruits of these three plants are taken in equal proportions and mixed with honey to treat long-term fever and loss of appetite. Fruits of these three plants are soaked in water, which is then drunk every morning to treat gastrointestinal disorders. The traditional Ayurvedic formulation consisting of a combination of fruits of these three plants is called “triphala.”

The leaves of 19 Japanese woody plants were also investigated for potential inhibitory effects against α-glucosidase in this study. Medical value of these plant species is not known in Japan. In addition, there have been no previous reports regarding *in vitro* α-glucosidase inhibitory activity of these plants.

1.9 Aims of dissertation

The aims of this study are summarized as follows:

To evaluate the antidiabetic potential of different extracts of the leaves from three Bangladeshi plants (Chapter 2).

To investigate the effects of different extracts of the leaves from 19 Japanese woody plants (Chapter 3).

To isolate and purify active compounds from the plant extracts that have inhibitory effects against α-glucosidase (Chapter 4).

Chapter 2

Inhibitory effects of Bangladeshi medicinal plant leaf extracts on α-glucosidase activity

Abstract

One of the best strategies in treatment of diabetes mellitus management involves control of postprandial hyperglycemia through enzymatic inhibition of starch degradation. *Emblica officinalis, Terminalia bellirica*, and *Terminalia chebula* are used as remedies in Ayurvedic medicine. In this study, methanol extracts of the leaves of these three plant species were screened for their α-glucosidase inhibitory potential activities in *vitro*. Methanol extract of *T. chebula* showed maximum inhibitory activity against yeast α-glucosidase with a half maximal inhibitory concentration (IC_{50}) of 15 µg/ml, followed by *T. bellirica* with IC_{50} value of 34 µg/ml and *E. officinalis* with a value of 50 µg/ml, compared with the standard drug, acarbose (IC_{50} value: 13 mg/ml). Rat intestinal sucrase inhibitory activity was also investigated. The methanol extracts of these three plants at 1 mg/ml showed considerable rat intestinal sucrase inhibitory activity. The hexane extract, ethyl acetate extract, butanol extract, and water extract from the three plants were screened for yeast α-glucosidase and rat intestinal sucrase inhibitory activity. Among the three plants, butanol extract exhibited potent α-glucosidase inhibitory activity. Methanol and butanol extracts of the three plants also showed excellent inhibitory activity against rat intestinal sucrase. *E. officinali*s, *T. bellirica*, and *T. chebula* are potential plant sources of α-glucosidase inhibitors that can be used to treat diabetes.

Keywords: α-glucosidase inhibitor, diabetes, *Emblica officinalis*, *Terminalia bellirica*, *Terminalia chebula*

2.1 Introduction

The incidence of diabetes is increasing worldwide. Saquib *et al*. (2012) reported that the number of diabetes patients in Bangladesh has increased from 3.8% in 1995 to 9% in 2010. The prevalence of diabetes is increasing in Bangladesh due to inadequate healthcare facilities in rural areas, lack of health consciousness, poor economic conditions, lifestyle changes caused by rapid urbanization, and changes in food habits. The Global Change Makers Program also indicated that the poor people in Bangladesh are suffering lifestyle-related diseases such as diabetes (Global Change Makers Program, 2013).

Although the numbers of diabetic patients are rapidly increasing, countermeasures have not been developed in Bangladesh. There are several reasons for the difficulty in coping with diabetes in Bangladesh, e.g., poverty, limitations of health care facilities, low capability of spending on health care expenses, lack of understanding of health condition, lack of education, lack of information about diabetes, inadequate opportunities for annual health checks and lack of knowledge prevent people in this country from taking modern medicines. Especially, facilities for diabetes treatment are not available in health centers, and nurse practitioners do not have sufficient training for early identification of prediabetes in Bangladesh (Islam *et al*., 2013).

Traditional medicines, which are commonly used in rural areas, represent one solution to these problems. Traditional medicine has a long history of use in Bangladesh, and has the advantage of reduced medical expenses. Rahman (2013) reported that large numbers of people use traditional medicines for various diseases, such as skin diseases, joint pain, diarrhea, cough, stomach problems, and diabetes. Ayurvedic traditional medicine has been used since ancient times in Bangladesh (Yoshida *et al*., 2016). Traditional medicine has been reported to show fewer side effects than allopathic medicines, and Vipula (2014) reported a good response to Ayurvedic treatment compared to allopathic treatment, with no side effects.

Traditional medicinal plants are also used to provide affordable treatments for diabetes mellitus with advances in many herbal medicinal health care systems in Bangladesh. Various types of medicinal plants and plant parts are used in traditional medicine for diabetes. In addition to traditional practitioners reporting good responses, their treatments can be validated scientifically based on the antidiabetic properties of

A. *Emblica officinalis* B.*Terminalia chebula* C. *Terminilia bellirica*

Figure 2.1 Plant leaves used in this study.

A: *E. officinalis* belongs to the family Euphorbiaceae. B: *T. chebula* belongs to the family Combretaceae. C: *T. bellirica* belongs to the family Combretaceae.

components of their medicinal plants. Grover *et al*. (2002) showed that antihyperglycemic agents are present in various plant extracts that have been used as traditional medicine.

In this study, we focused on three plants, *Emblica officinalis*, *Terminalia bellirica*, and *Terminalia chebula* (Fig. 2.1), which are widely distributed southern India, Pakistan, Bangladesh and other South-East Asian countries and have been extensively used in traditional medicine in Bangladesh and the Indian subcontinent. The traditional herbal formulation consisting of a combination of fruits of these three plants called "triphala" is very popular in traditional Ayurvedic medicine, and has been used for thousands of years against various types of disease. Mukherjee *et al*. (2006) reported that "triphala and triphala mixture" follows a standardized ayurvedic formula, which has been clinically tested and shown to exhibit good results against constipation and appetite problems.

These three plants are well known as individual herbal remedies. *E. officinalis* has been reported to possess antiinflammatory (Golechha *et al*., 2014), antioxidant (Scartezzini *et al*., 2006), hepatoprotective (Jose *et al*., 2000), antipyretic and analgesic activities (Perianayagam *et al*., 2004). *T. chebula* has been reported to show anticancer (Ahuja *et al*., 2013), antiviral (Kim *et al*., 2001), antibacterial (Kannan *et al*., 2009), antioxidant (Cheng *et al*., 2003), and wound healing activities (Singh *et al*., 2009). *T. bellirica* has been studied for its antimicrobial (Devi *et al*., 2014), anti-HIV-1, antimalarial, antifungal (Valsaraj *et al*., 1997), analgesic, and antipyretic activities (Sharma *et al*., 2010).

Moreover, Sabu and Kuttan (2002) reported that oral administration of an extract of *E. officinalis, T. bellirica,* and *T. chebula* fruits effectively decreased the serum glucose level in diabetic rats. *T. chebula* seed extracts showed a remarkable decrease in blood glucose level by increasing insulin secretion in both short and long term studies (Rao and Nammi, 2006).

The therapeutic potentials of various parts of the above three plants from different regions have been evaluated in previous studies. However, the antidiabetic potential of the leaves collected from Bangladesh has not been reported. While the fruits and seeds of *E. officinalis, T. chebula, and T. bellirica* have been reported to show antihyperglycemic effects, there have been no studies regarding the α-glucosidase inhibitory activity of leaves of these three plants collected from Bangladesh. The present study was designed to evaluate the antidiabetic potentials of different extracts of these three plant leaves through monitoring of the inhibitory effects on yeast and rat intestinal α-glucosidase.

α-Glucosidases, which include maltase, maltase-glucoamylase, etc., are the key enzymes. The α-glucosidase from the yeast, *Saccharomyces cerevisiae* is commonly used to search for biologically active compounds with inhibitory effects on the enzyme in *vitro*. Hogan *et al*. (2010) used yeast α-glucosidase as a model for isolation and identification of inhibitory substances from natural products. The *p*-nitrophenyl-α-D-glucopyranoside was used as a synthetic substrate in the assay of yeast α- glucosidase, which catalyzes glucose and p-nitrophenol. Yeast α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside substrates were developed for analysis of maltase inhibitory activity. Starch is the most important carbohydrate in the human body, and is catalyzed into maltose by α-amylase, which in turn is catalyzed into glucose by maltase in the brush-border surface membrane of the small intestine.

Sucrose is the most commonly used sugar all over the world, and is degraded into glucose and fructose by the α-glucosidase, sucrase. Rat intestinal acetone powder has been used as a source of sucrase for in *vitro* assays with sucrose as the substrate. Ohta *et al*. (2002) reported that the rat intestinal acetone powder α-glucosidase closely mimics that in the mammalian system in *vivo* and may be a superior model for use in studies to develop methods for the control of postprandial blood glucose. Enzymes from different

sources catalyze different substrates. Therefore, we used two types of α-glucosidase, i.e., yeast α-glucosidase and rat intestinal α-glucosidase, in this study.

Diabetes mellitus is a group of serious metabolic disorders associated with high blood glucose levels due to a failure of the body to produce insulin properly. At present, three main types of diabetes are known, i.e., type I insulin-dependent diabetes, type II non-insulin-dependent diabetes, and gestational diabetes (International Diabetes Federation, 2013). Type II has the highest prevalence rate, and the control of postprandial blood glucose level is important in its treatment. The inhibition of α-glucosidase is essential to delay carbohydrate digestion and absorption, reduce blood glucose levels, and finally control postprandial hyperglycemia. Acarbose, miglitol, and voglibose are well-known clinically approved inhibitors of α-glucosidase activity (Van de Laar, 2008). However, these α-glucosidase inhibitors have number of undesirable side effects, including gastrointestinal problems (Cheng *et al*., 2005). Therefore, natural α-glucosidase inhibitors that are safe, effective, and have no or only minor side effects are required.

2.2 Materials and Methods

2.2.1 Plant collection and extraction

The leaves of the three plant species were collected from the Bangladesh Agricultural University, packed in the newspaper, and dried in the sun. The dried samples were crushed in a blender, and then boiled in methanol at 60ºC -70ºC three times. Boiled leaf extracts were dried under reduced pressure using a rotary evaporator. These dried methanol extracts were dissolved in 30% hexane/70% methanol solution and successively partitioned with the same volume of hexane, ethyl acetate, butanol, and water by adding each solution as shown in Fig. 2.2. The solvents were again evaporated and the remaining substances were used as the test materials. The methanol extracts and their partitioned fractions were monitored for inhibitory effects on α-glucosidase activity.

Generally, plant bioactive compounds consist of various classes of chemicals, such as alkaloids, glycosides, lignins, tannins, terpenoids, etc. The methanol extracts of the plants were used because many molecules, including fatty acids, water-soluble materials, and other bioactive compounds, are soluble in methanol. The major biological compound, bartogenic acid, was identified from the methanol extract of *Barringtonia racemosa* seed (Gowri *et al*., 2007). Therefore, methanol is important in screening for biologically active compounds, and was used in the present study.

2.2.2 Yeast α-glucosidase inhibitory assay

The inhibitory activity of yeast α-glucosidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was determined by the method of Babu *et al*. (2004) with slightly

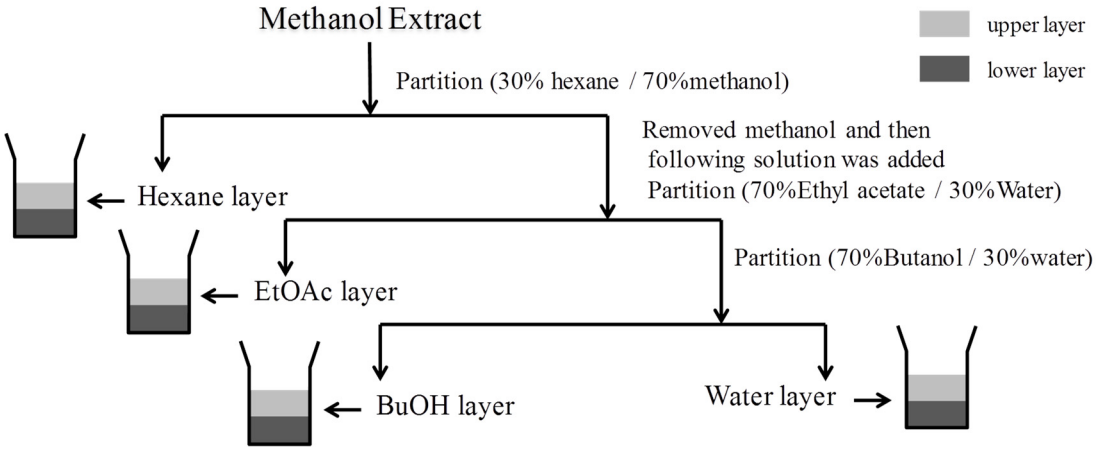

Fig. 2.2 Separation by partition scheme with several solvents. The upper layers are hexane, ethyl acetate, and butanol. The lower layers are water.

modifications. Aliquots of 20 µl of the test samples dissolved in methanol at 10 mg/ml were serially diluted in 96-well microtiter plates. The plant extracts diluted in10 µl of phosphate buffer 0.1 mol/l (pH 6.8) and 150 µl (5 mmol/ml) of *p*-nitrophenyl-α-D-glucopyranoside (PNPG) were added to each well. The reaction was started by addition of 20 µl (5 µg/ml) of the enzyme to the reaction mixture in the 96-well plates. Individual blanks where the substrate was replaced with 40 µl of phosphate buffer were prepared to correct for background absorbance. The controls contained 20 µl of phosphate buffer in place of the test sample, while the leaf extract was replaced with acarbose in positive controls. All determinations were performed in triplicate. The changes in absorbance at 405 nm (A_{405}) were recorded at 1-minute intervals for 10 minutes, and the percentage inhibition was estimated from the slope using the following equation:

Inhibition % = (1- slope of test sample/slope of control) ×100.

The concentration that gave the half-maximal response (IC_{50}) was determined from the sample concentration vs. percentage inhibition rate. α-Glucosidase inhibitory activity was expressed as inhibition % and IC_{50} value, with a lower IC_{50} value was indicating higher inhibitory activity.

2.2.3 Rat intestinal α-glucosidase (sucrase) inhibitory assay

Rat intestinal α-glucosidase inhibitory activity was determined by a modification of the method of Babu *et al*. (2004). Aliquots of 20 µl of plant extract test samples dissolved in methanol at 10 mg/ml were added to each well of 96-well plates. Then, 150 µl of 5 mg/ml saccharose was added to each well. Rat intestinal acetone powder (Sigma-Aldrich, St. Louis, MO) was added at 100 mg/ml to phosphate buffer 0.1 mol/l (pH 6.8) and sonicated for 5 minutes. The suspension was centrifuged at 2500 rpm for 5 minutes to remove particulate matter, and the resulting supernatant was used as the enzyme solution. The reaction was initiated by addition of 30 µl of the enzyme to the reaction mixture in 96-well plates. The reaction mixture was incubated at 37ºC for 30 minutes followed by 70ºC for 3 minutes on a heating block to stop the reaction. The reaction mixture was cooled to room temperature for 10 minutes and aliquots of 20 µl were transferred to another plate. The reaction was started by addition of 150 µl of reagent (Glucose C2; Wako) and cooled to 25ºC for 15 minutes. Individual blanks where the substrate was replaced with 50 µl of phosphate buffer were prepared to correct for

background absorbance. The controls contained 20 µl of phosphate buffer in place of the test sample, while the leaf extract was replaced with acarbose in positive controls. All determinations were performed in triplicate. The changes in absorbance at 492 nm (A_{492}) were recorded at 1-minute. The percentage inhibition was estimated using the following equation:

Inhibition % = (1 - (absorbance of test sample-absorbance of blank) / (absorbance of control-absorbance of blank)) ×100.

2.2.4 Statistical analysis

The effects of crude methanol extracts (Fig. 2.3) and fractionated extracts (Fig. 2.4) of the leaves from three medicinal plants, *E. officinalis*, *T. bellirica*, and *T. chebula* were examined by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using SPSS version 16.0 for windows (SPSS Inc., Chicago, IL). In all analyses, $p < 0.01$was taken to indicate statistical significance.

2.3 Results

E. officinalis yielded the highest percentage (18%) of methanol extract from 50 g of plant material (Table 2.1), with *T. chebula* and *T. bellirica* showing methanol extract yields of 15% and 12%, respectively (Table 2.1). The methanol extracts were screened for their α-glucosidase inhibitory activities. The methanol extracts of the three plants showed significant in *vitro* α-glucosidase inhibitory activity compared with acarbose (13 mg/ml), with *T. chebula* showing the highest inhibitory activity (IC_{50} = 15 µg/ml), followed by *T. bellirica* (IC_{50} = 34 µg/ml), and *E. officinalis* (IC_{50} = 50 µg/ml) (Table 2.2).

The hexane fraction of *T. chebula* leaves was inactive, while the ethyl acetate, butanol, and water fractions showed α-glucosidase inhibitory activity. The *T. chebula* butanol fraction showed a strong inhibitory effect with an IC_{50} value of 10 µg/ml (Table 2.3). The hexane fraction of *E. officinalis* leaf extract showed no inhibitory activity against α-glucosidase. The butanol and ethyl acetate fractions of *E. officinalis* showed almost the same inhibitory activities, with IC_{50} values of 18 µg/ml and 19 µg/ml, respectively (Table 2.3). The hexane fraction of *T. bellirica* leaves was also less active than the ethyl acetate, butanol, and water fractions, with IC_{50} values for the butanol and ethyl acetate fractions of 12 µg/ml and 18 µg/ml, respectively (Table 2.3).

The inhibitory activities of the methanol extracts (12.5 µg/ml) and fractionated extracts (10 µg/ml) from the three plants against yeast α-glucosidase were analyzed and

Table 2.1 Efficiency of methanol extraction from the leaves of three Bangladeshi medicinal plants

Scientific Name	Family name	Leaf weight (gm)	Quantity of methanol extract (gm)	Extract rate (%)
Emblica officinalis	Euphorbiaceae	50	9	18
Terminalia chebula	Combretaceae	30	5	15
Terminalia bellirica	Combretaceae	26	3	12

Percentage extract yield (w/w) was calculated as (dry extract weight/dry starting material weight) × 100.

Table 2.2 Effects of methanol extracts from the leaves of three medicinal plants against yeast α-glucosidase.

Plant Name	IC_{50} µg/ml
Emblica officinalis	50
Terminalia chebula	15
Terminalia bellirica	34
Acarbose (positive control:)	13,000

Acarbose was used as a positive control.

IC_{50}: Concentration of the antagonist that inhibited the enzyme reaction by 50%.

Table 2.3 Inhibitory effects of fractionated extracts of three medicinal plants on yeast α-glucosidase activity

Plant Name	IC_{50} µg/ml			
	Hexane extract	Ethyl acetate extract	Butanol extract	Water extract
Emblica officinalis	NA	19	18	37
Terminalia chebula	NA	26	10	10
Terminalia bellirica	102	18	12	33
Acarbose (positive control)	13,000			

Acarbose was used as a positive inhibitory molecule.
IC_{50}: Concentration of the antagonist that inhibited the enzyme reaction by 50%.
NA: No activity

the results are shown in Figures 2.3 and 2.4, respectively. There were significant differences in the effects of the methanol extracts between the plant species, with that of *T. chebula* showing the maximum inhibitory effect (46%) followed by *E. officinalis* (32%) and *T. bellirica* (30%) (Fig. 2.3). There were no significant differences in the inhibitory effects of ethyl acetate extracts among the three plant species (Fig. 2.4). The butanol extracts of *T. chebula* and *T. bellirica* showed similar inhibitory effects (50% and 46%, respectively), while *E. officinalis* showed a weaker effect (32%) (Fig. 2.4). The water extract of *T. chebula* showed the greatest inhibitory effect (50%), with *E. officinalis* and *T. bellirica* are showing lower inhibitory effects (21% and 23%, respectively) (Fig. 2.4).

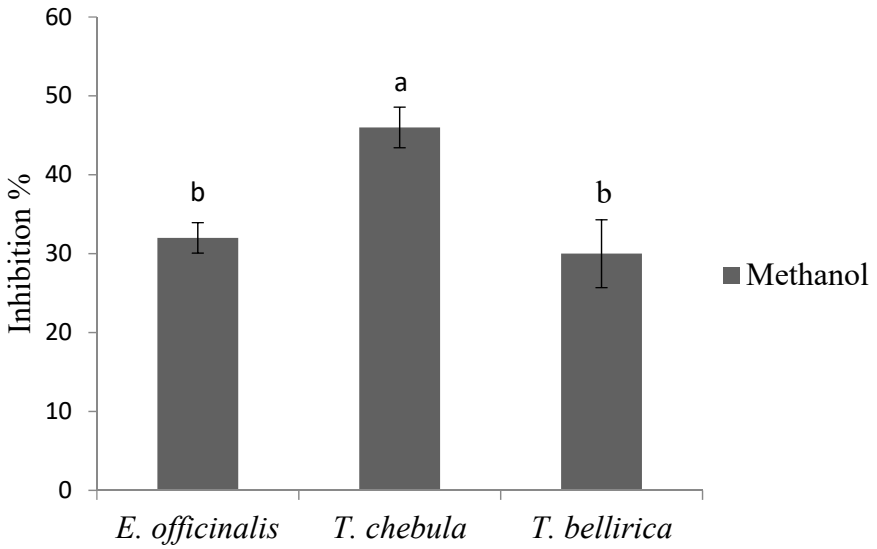

Fig. 2.3 Effects of methanol extracts of the leaves from three medicinal plants on yeast α-glucosidase activity (12.5 µg/ml).
The same letters above the bars in the graph indicate that the mean did not differ significantly, while different letters indicate significant variation at $P < 0.01$. The values are expressed as means ± standard deviation, n =3.

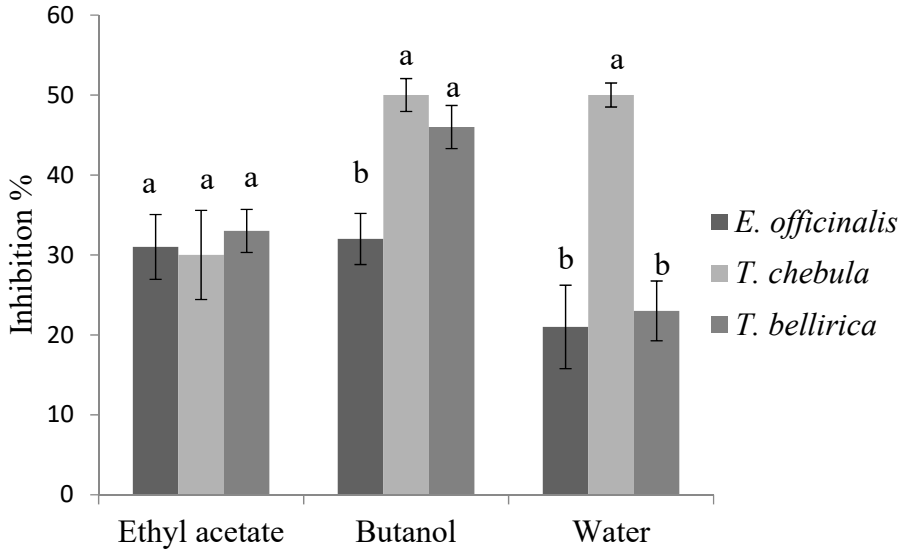

Fig. 2.4 Inhibitory effects of fractionated extracts of the leaves from three medicinal plants on yeast α-glucosidase activity (10 µg/ml).

The same letters above the bars in the graph indicate that the mean did not differ significantly, while different letters indicate significant variation at $P < 0.01$. The values are expressed as means ± standard deviation, n =3.

In this study, the inhibitory effects of leaf extracts from the three plant species against sucrase activity were determined and compared with that of acarbose (91%, Table 2.4). The methanol extracts of *E. officinalis*, *T. chebula*, and *T. bellirica* leaves at 1 mg/ml

Table 2.4 Inhibitory effects of extracts of the leaves from three medicinal plants on rat intestinal sucrase and yeast α-glucosidase.

Plant Name	Extract Type	% of rat intestinal sucrase inhibitory effect	% of yeast α-glucosidase inhibitory effect
		1mg/ml	200 µg/ml
Emblica officinalis	Methanol	81 ± 7	95 ± 1
	Hexane	33 ± 3	22 ± 5
	Ethyl acetate	49 ± 7	95 ± 1
	Butanol	58 ± 4	98 ± 1
	Water	50 ± 2	83 ± 2
Terminalia chebula	Methanol	89 ± 4	90 ± 1
	Hexane	39 ± 11	9 ± 3
	Ethyl acetate	54 ± 10	68 ± 0
	Butanol	67 ± 14	97 ± 1
	Water	62 ± 10	94 ± 1
Terminalia bellirica	Methanol	70 ± 9	89 ± 2
	Hexane	27 ± 6	65 ± 4
	Ethyl acetate	41 ± 1	94 ± 0
	Butanol	50 ± 5	98 ± 0
	Water	38 ± 8	94 ± 1
Acarbose (positive control)		91 ± 3	5 ± 4

The values are expressed as means ± standard deviation, n =3.

in the reaction mixtures showed significant inhibitory effects against sucrase enzyme activity (Table 2.4), with that of *T. chebula* showing the greatest effect (89%). We also investigated the inhibitory effects of hexane, ethyl acetate, butanol, and water extracts of the leaves from these plants against sucrase activity. The butanol extract of *E. officinalis* and water extract of *E. officinalis* were shown to inhibit the enzyme activity by 58% and 50%, respectively (Table 2.4). However, the hexane extract of *E. officinalis* showed only a weak inhibitory effect (33%) on sucrase activity, while acarbose as a positive control showed 91% inhibition of the enzyme activity at a concentration of 1 mg/ml (Table 2.4).

Ethyl acetate, butanol, and water fractions of *T. chebula* leaves showed the highest sucrase inhibitory effects (54%, 67%, and 62%, respectively), and the weakest effect was observed for the hexane extract of *T. chebula* (39%, Table 2.4). The butanol extract of *T. bellirica* leaves showed an inhibitory effect of 50%, while the hexane, ethyl acetate, and water extracts showed lower inhibitory activities of 27%, 41%, and 38%, respectively (Table 2.4).

2.4 Discussion

Although acarbose is a well-known antidiabetic medication, Oki *et al*. (1999) reported that acarbose did not inhibit yeast α-glucosidase activity, while it showed inhibitory effects on rat, rabbit, and pig small intestinal α-glucosidase activities. Kim *et al*. (2004) reported a similar result where pine bark extracts showed strong inhibitory effects against yeast α-glucosidase, while the standard drug, acarbose, showed no inhibitory effect on yeast α-glucosidase activity. These observations were compatible with those of the present study, in which acarbose showed only a weak inhibitory effect against yeast α-glucosidase with an IC_{50} value of 13 mg/ml, while the three plant extracts showed strong inhibitory effects against the activity of this enzyme (Table 2.3). In this study, acarbose had an IC_{50} value of 13 mg/ml for yeast α-glucosidase at 5 μg/ml in the reaction mixture, whereas Shai *et al*. (2011) reported that acarbose had an IC_{50} value of 1.5 mg/ml for yeast α-glucosidase at 0.5 mg/ml in the reaction mixture. These results indicated that the different inhibitory effect of acarbose on yeast α-glucosidase, which may have been due to the use of different concentration of the enzyme. Shai *et al*. (2011) also mentioned that the different concentrations of the enzyme are responsible for different IC50 value of acarbose.

The methanol and butanol extracts of the leaves from the three plant species, *E. officinalis*, *T. bellirica*, and *T. chebula*, showed stronger inhibitory effects against yeast α-glucosidase than rat intestinal sucrase (Table 2.4). At 200 μg/ml, methanol and butanol extracts of *T. chebula* inhibited yeast α-glucosidase activity by 90% and 97%, respectively, while at 1 mg/ml, methanol and butanol extracts of the effects of that inhibited on rat intestinal sucrase were lower at 89% and 67%, respectively. This result was supported by those of Shai *et al.* (2011), who found that acetone extracts of different plants had stronger inhibitory effects against yeast α-glucosidase than mammalian α-glucosidase. Havsteen (1983) classified flavonoids into six groups based on the variation of benzopyran and phenyl groups and their ring and linkage sites, i.e., flavone, flavonol, flavanone, isoflavone, flavan-3-ol, and anthocyanidin. Tadera *et al*. (2006) reported that the inhibitory effects on yeast α-glucosidase and rat intestinal α-glucosidase differed according to the chemical structure of flavonoids based on the presence of absence of the OH group at the C-3 position of the flavone (quercetin > luteolin; kaempferol > apigenin),

hydroxyl substitution on the B ring (flavonol: myricetin = quercetin > kaempferol; flavone: luteolin > apigenin), linkage of the B-3 position (isoflavone and flavone groups: genistein > apigenin), and 1,2- and 3,4-double bonds and lack of 4-CO of cyanidin, which is responsible for significant inhibition of yeast α-glucosidase. On the other hand, the anthocyanidin group and isoflavone group were associated with weak inhibition of rat intestinal α-glucosidase activity.

In the present study, butanol extracts of *T. chebula* and *T. bellirica* showed stronger inhibitory effects against yeast α-glucosidase activities with IC_{50} values of 10 µg/ml and 12 µg/ml, respectively (Table 2.3), than against rat intestinal α-glucosidase (Table 2.4). This result was supported by the previous report of Tadera *et al*. (2006), who also found that yeast α-glucosidase was strongly inhibited by flavonoids with IC_{50} values < 15µM, and that rat small intestinal α-glucosidase was weakly inhibited by many flavonoids and faintly by the isoflavone and anthocyanidin groups. Sim *et al*. (2010) reported that the substrate specificity is responsible for different reactions because the differences of active site of enzyme between N-terminal maltase-glucoamylase and N-terminal sucrase-isomaltase where maltose could be hydrolyzed efficiently by both N-terminal sucrase-isomaltase and N-terminal maltase-glucoamylase, because N-terminal sucrase-isomaltase has a narrow +1 subsite which accommodates both its α-1,4 (maltose) and α-1,6 (isomaltose) substrates, but N-terminal maltase-glucoamylase has a wide +1 subsite, which is specific for the α-1,4 substrate. The present study results showed different reactions against yeast α-glucosidase and rat intestinal α-glucosidase using the different extract of three plant leaves, these different reactions might be due to the substrate specificity, which was demonstrated by Sim *et al.* (2010).

The methanol extracts of *T. chebula*, *T. bellirica*, and *E. officinalis* had IC_{50} values for yeast α-glucosidase of 15 µg/ml, 34 µg/ml, and 50 µg/ml, respectively (Table 2.2). On the other hand, Prihantini *et al.* (2014) reported that the methanol extract of *Distylium racemosum* showed a strong inhibitory effect on yeast α-glucosidase with an IC_{50} value of 22.6 ± 1.9 µg/ml, followed by *Acer mono Maxim* and *Elaeocarpus sylvestris* with IC_{50} values of 56.5 ± 0.9 µg/ml and 74.4 ± 0.9 µg/ml, respectively. These results indicated that the α-glucosidase inhibitory activity of methanol extract of *T. chebula* was much stronger than that of *D. racemosum*, which has been identified as a α-glucosidase inhibitor. Nampoothiri *et al.* (2011) reported that *T. bellirica* fruit extract showed stronger

inhibitory activity against α-glucosidase than *E. officinalis* fruit extract (IC_{50} values 0.75 µg/ml and 1.0 µg/ml, respectively). These results indicated higher inhibitory activities than those seen in the present study, which may have been due to the use of different parts of the plants (i.e., fruit vs. leaf extracts, respectively). In addition, Oboh *et al.* (2014) reported that different parts of *Persea americana* (avocado pear) plants showed different α-glucosidase inhibitory activities.

These results of the present study indicated that the methanol extract of *T. chebula* leaves had the highest inhibitory potency against rat intestinal sucrase inhibitory activity (89%), followed by those of *E. officinalis* (81%) and *T. bellirica* (70%) (Table 2.4), while Sabu and Kuttan (2002) reported that the methanol extract of *T. bellirica* fruits showed maximum inhibitory activity (52.74%), followed by those of *T. chebula* (50.98%) and *E. officinalis* (29.52%). These discrepancies may have been due to differences in the experimental approach, because our experiments were performed in *vitro*, whereas Sabu and Kuttan (2002) performed in *vivo* experiments. In the present study, ethyl acetate extract of *T. chebula* leaves showed 54% inhibition (1 mg/ml) against rat intestinal sucrase, while Kim *et al.* (2011) reported that a high dose of the ethyl acetate portion of ethanolic extract of *T. chebula* fruit (500 mg/kg) reduced the levels of blood glucose. This result showed good agreement with those of the present study.

The results of the present study indicated that the methanol and butanol extracts of *T. chebula* leaves and butanol extracts of *T. bellirica* leaves showed maximum inhibitory effects on yeast α-glucosidase activity. Methanol and butanol extracts of the leaves of the three plants, *T. chebula*, *T. bellirica*, and *E. officinalis*, showed the strong inhibitory effects on rat intestinal sucrase activity. Thus, the leaves of these three plant species may contain antihyperglycemic compounds. Further structural elucidation and characterization are required to identify for identifying the biologically active constituents of these extracts, and in *vivo* studies are also necessary to confirm these observations.

2.5 Conclusions

The leaf extracts of three Bangladeshi medicinal plants *T. chebula*, *T. bellirica*, and *E.* officinalis, showed significant inhibitory effects against α-glucosidase and sucrase activities. Butanol and methanol extracts of *T. chebula* leaves and butanol extract of *T. bellirica* leaves showed significant inhibitory effects on carbohydrate digestive enzymes, which may function in controlling postprandial glucose levels. However, further studies are needed to determine their potential efficacy in the treatment of diabetes.

Chapter 3

Inhibitory effects of Japanese plant leaf extracts on α-glucosidase activity

Abstract

In this study, methanol extracts of the leaves of nineteen Japanese plant species for which there have been no previous reports regarding *in vitro* α-glucosidase activities, were tested for their potential α-glucosidase inhibitory activities. Five plants, *Quercus phillyraeoides*, *Mallotus japonicus*, *Sapium sebiferum*, *Elaeocarpus sylvestris* var. *ellipticus*, and *Myrica rubra*, showed high inhibitory activity against α-glucosidase with IC_{50} values in the range of 22 – 92 μg/ml. The methanol extracts of these five plants at 1 mg/ml showed significant inhibitory activity against rat intestinal sucrase. The hexane, ethyl acetate, butanol, and water extracts from the five plants were also screened for yeast α-glucosidase and rat intestinal sucrase inhibitory activity. The extracts of *Q. phillyraeoides* leaves (ethyl acetate and butanol extracts), *M. japonicas* leaves (ethyl acetate and butanol extracts), and *E. sylvestris* var. *ellipticus* leaves (ethyl acetate and butanol extracts) showed excellent inhibitory effects against yeast α-glucosidase. Methanol, ethyl acetate, and butanol extracts of the five plants also exhibited potent inhibitory effects against rat intestinal sucrase. Due to their inhibitory effect, the leaves of these five plant species were selected for further investigation to isolate and identify the active constituent(s) responsible for the potential antidiabetic activity.

Keywords**:** α-glucosidase inhibitor, Diabetes mellitus

3.1 Introduction

Diabetes mellitus is one of the most serious chronic metabolic diseases. The number of diabetes patients is increasing in the world not only in developed countries but also in developing countries due to changes in lifestyle and food habits. The total diabetic population is predicted to increase from 171 million in 2000 to 366 million in 2030 (Wild *et al*., 2004). In Japan, the prevalence of type 2 diabetes is increasing in both adults and children; about 13.5% of the Japanese population had type 2 diabetes in 2009 (Neville *et al*., 2009). Therefore, it is clear that diabetes is a very serious concern.

Blood glucose levels in the human body are increased due to diabetes, often leading to various complications, such as cardiovascular disease, eye problems, kidney disease, cerebrovascular disease, and limb amputation. Therefore, control of postprandial blood glucose levels is important for the treatment of diabetes. Diet and exercise are recommended to control blood glucose levels. Although diet and exercise are recommended for diabetes management. In most cases, medications are needed for the treatment of diabetes. Neville *et al*. (2009) reported that 51.4% of Japanese people with diabetes take oral antidiabetic drugs, while only 25.4% control diabetes by diet. Acarbose and voglibose are common α-glucosidase inhibitors used in the treatment of diabetes (Playford *et al*., 2013; Saito *et al*., 1998). However, they often cause severe gastrointestinal side effects. Therefore, an investigation of new α-glucosidase inhibitors from natural resources has become an attractive approach for the treatment of diabetes.

A wide variety of active components of plants have been identified that have effects on human health. From ancient times, plants have fulfilled our dietary requirements and have also been used for various medical purposes (Kim and Kwon, 2011). Medicinally active compounds, including carbohydrate digestive enzyme (α-glucosidase and sucrase) inhibitors, are widely distributed in plants (Mai *et al*., 2007). Some plants have been shown to contain α-glucosidase inhibitors (Wu *et al*., 2011; Alagesan *et al*., 2012; Chu *et al*., 2014).

In this study, the leaves of Japanese plant species were examined for α-glucosidase inhibitory activity. There have been no previous reports regarding such components of these plant species. Chowdhury *et al*. (2009) reported that among plant parts, the leaves are used most commonly against diseases (37%). Chowdhury *et al*.

(2009) also mentioned that most researchers use the leaves of plants as test materials to screen for active components. Halim *et al*. (2007) suggested that the common use of leaves was due to the ease of harvesting from plants. The present study was designed to investigate the antidiabetic effects of different extracts of the leaves of nineteen Japanese woody plant species on yeast α-glucosidase (maltase) and rat intestinal sucrase.

3.2 Materials and methods

3.2.1 Plant collection and extraction

The leaves of the nineteen plants species (scientific and family names along with extract yields are listed in Table 3.1) were collected from Kochi University, Monobe campus, Japan. The plant extraction procedure was described in Chapter 2.

3.2.2 Assay

The yeast and rat intestinal α-glucosidase inhibitory activity assays were performed as described Chapter 2.

Table 3.1 Efficiency of methanol extraction from the leaves of nineteen Japanese plants

Scientific Name	Family Name	Leaf weight (g)	Quantity of methanol extract (g)	Extract yield (%, w/w)
Robinia pseudoacacia	Fabaceae	10	2.44	24.4
Morus bombycis	Moraceae	10	1.68	16.8
Rubus hirsutus	Rosaceae	20	4.22	21.1
Broussonetia kazinoki	Moraceae	20	4.76	23.8
Celtis sinensis var. *japonica*	Cannabaceae	15	2.46	16.4
Sapium sebiferum	Euphorbiaceae	5	1.86	37.2
Prunus jamasakura	Rosaceae	20	3.39	16.95
Prunus × yedoensis	Rosaceae	10	2.13	21.3
Cinnamomum camphora	Lauraceae	15	2.57	17.1
Elaeocarpus sylvestris var. *ellipticus*	Elaeocarpaceae	30	6.72	22.4
Zelkova serrata	Ulmaceae	10	1.83	18.3
Melia azedarach var. *subtripinnata*	Meliaceae	20	4.62	23.1
Mallotus japonicus	Euphorbiaceae	15	4.84	32.3
Hedera rhombea	Araliaceae	10	2.37	23.7
Quercus phillyraeoides	Fagaceae	20	3.60	36
Lonicera japonica	Caprifoliaceae	15	1.92	12.8
Myrica rubra	Myricaceae	20	2.97	14.9
Aphananthe aspera	Cannabaceae	10	1.76	17.6
Hibiscus syriacus	Malvaceae	10	2.20	22

Percentage extract yield (w/w) was calculated as (dry extract weight/dry starting material weight) × 100

3.3 Results

The methanol extracts of nineteen plants were investigated for their α-glucosidase activities. Extract concentrations of 200 μg/ml and 50 μg/ml were used for preliminary investigations (Table 3.2). Five plants (*E. sylvestris* var. *ellipticus*, *M. japonicus*, *M. rubra*, *Q. phillyraeoides*, and *S. sebiferum*) showed high levels of inhibitory activity against α-glucosidase (Table 3.2). Therefore, these five plants were analyzed comparatively with regard to their α-glucosidase inhibitory activities. The methanol extracts of the five plants showed significant in *vitro* α-glucosidase inhibitory activity compared with acarbose (13 mg/ml). Among the five plants, *Q. phillyraeoides* and *E. sylvestris* var. *ellipticus* showed the same inhibitory effects (IC_{50} = 22 μg/ml and 22 μg/ml, respectively), followed by *S. sebiferum* (IC_{50} = 42 μg/ml), *M. japonicus* (IC_{50} = 52 μg/ml), and *M. rubra* (IC_{50} = 92 μg/ml) (Table 3.3).

The hexane and water fractions of *Q. phillyraeoides* leaves had little activity, while ethyl acetate and butanol fractions showed stronger α-glucosidase inhibitory activities. The *Q. phillyraeoides* ethyl acetate fraction showed a strong inhibitory effect with an IC_{50} value of 4 μg/ml (Table 3.4). The hexane and water fractions of *M. japonicus* leaves showed no inhibitory activity against α-glucosidase (Table 3.4). The ethyl acetate

Table 3.2 Effects of methanol extracts of the leaves of nineteen plants against yeast α-glucosidase

Scientific Name	Inhibition %	
	200 μg/ml (%)	50 μg/ml (%)
Robinia pseudoacacia	93 ± 1	15 ± 3
Morus bombycis	18 ± 2	1 ± 2
Rubus hirsutus	85 ± 1	20 ± 3
Broussonetia kazinoki	5 ± 8	1 ± 8
Celtis sinensis var. *japonica*	75 ± 4	4 ± 8
Sapium sebiferum	62 ± 1	53 ± 3
Prunus jamasakura	12 ± 2	6 ± 9
Prunus × *yedoensis*	17 ± 8	11 ± 2
Cinnamomum camphora	79 ± 2	6 ± 3
Elaeocarpus sylvestris var. *ellipticus*	65 ± 2	60 ± 5
Zelkova serrata	6 ± 12	7 ± 1
Melia azedarach var. *subtripinnata*	99 ± 1	91 ± 2
Mallotus japonicus	7 ± 9	3 ± 9
Hedera rhombea	82 ± 0	27 ± 0
Quercus phillyraeoides	30 ± 7	0 ± 1
Lonicera japonica	21 ± 2	0 ± 1
Myrica rubra	95 ± 6	11 ± 2
Aphananthe aspera	18 ± 2	1 ± 9
Hibiscus syriacus	86 ± 1	48 ± 2

Table 3.3 Inhibitory effects of fractionated extracts of five medicinal plants on α-glucosidase activity.

Plant Name	IC_{50} μg/ml
Quercus phillyraeoides	22
Mallotus japonicus	52
Sapium sebiferum	42
Elaeocarpus sylvestris var. *ellipticus*	22
Myrica rubra	92
Acarbose (positive control:)	13,000

Acarbose was used as a positive control.

IC_{50}: Concentration of the antagonist that inhibited the enzyme reaction by 50%.

and butanol fractions of *M. japonicus* leaves showed significant inhibitory activities, with IC_{50} values of 10 μg/ml and 25 μg/ml, respectively (Table 3.4). The hexane and water fractions of *S. sebiferum* leaves were also less active than the ethyl acetate and butanol fractions, the latter of which had IC_{50} values of 14 μg/ml and 16 μg/ml, respectively (Table 3.4). The hexane fraction of *E. sylvestris* var. *ellipticus* leaves was inactive, while the ethyl acetate, butanol, and water fractions showed α-glucosidase inhibitory activities, with IC_{50} values of 6 μg/ml, 7 μg/ml, and 9 μg/ml, respectively (Table 3.4). The hexane fraction of *M. rubra* leaves was inactive. The ethyl acetate fraction of *M. rubra* was less active than the butanol and water fractions, the latter of which had IC_{50} values of 15 μg/ml and 8 μg/ml, respectively (Table 3.4).

In this study, the inhibitory effects of leaf extracts from the five plant species against sucrase activity were determined and compared with that of acarbose (91%, Table 3.5). Methanol extracts of *E. sylvestris* var. *ellipticus*, *M. japonicus*, *M. rubra*, *Q. phillyraeoides*, and *S. sebiferum* leaves at 1 mg/ml in the reaction mixtures showed strong

Table 3.4 Inhibitory effects of fractionated extracts of five medicinal plants on yeast α-glucosidase activity

Plant Name	IC_{50} μg/ml			
	Hexane extract	Ethyl acetate extract	Butanol extract	Water extract
Quercus phillyraeoides	86	4	6	83
Mallotus japonicus	NA	10	25	NA
Sapium sebiferum	65	14	16	135
Elaeocarpus sylvestris var. *ellipticus*	NA	6	7	9
Myrica rubra	NA	84	15	8
Acarbose (positive control)	13,000			

Acarbose was used as a positive inhibitory molecule.

IC_{50}: Concentration of the antagonist that inhibited the enzyme reaction by 50%.

NA: No activity

Table 3.5 Inhibitory effects of extracts of the leaves of five plants on rat intestinal sucrase and yeast α-glucosidase.

Plant Name	Extract Type	% of rat intestinal sucrase inhibitory effect	% of yeast α-glucosidase inhibitory effect
		1mg/ml	200 µg/ml
Quercus phillyraeoides	Methanol	90 ± 4	99 ± 0.6
	Hexane	43 ± 21	69 ± 6
	Ethyl acetate	63 ± 6	100 ± 0
	Butanol	57 ± 18	99 ± 0
	Water	41 ± 7	81 ± 8
Mallotus japonicus	Methanol	73 ± 9	86 ± 2
	Hexane	35 ± 2	20 ± 3
	Ethyl acetate	57 ± 15	99 ± 0
	Butanol	67 ± 11	86 ± 1
	Water	50 ± 5	40 ± 1
Elaeocarpus sylvestris var. *ellipticus*	Methanol	77 ± 1	65 ± 3
	Hexane	13 ± 5	12 ± 3
	Ethyl acetate	70 ± 5	91 ± 2
	Butanol	62 ± 2	83 ± 6
	Water	27 ± 10	94 ± 2
Myrica rubra	Methanol	76 ± 0	82 ± 0
	Hexane	30 ± 6	28 ± 6
	Ethyl acetate	41 ± 8	73 ± 2
	Butanol	48 ± 4	94 ± 1
	Water	28 ± 9	99 ± 0
Sapium sebiferum	Methanol	69 ± 8	62 ± 4
	Hexane	51 ± 18	81 ± 0.4
	Ethyl acetate	63 ± 17	92 ± 1
	Butanol	76 ± 6	99 ± 2
	Water	56 ± 17	58 ± 1
Acarbose (positive control)		91 ± 3	5 ± 4

The values are expressed as means ± standard deviation, n =3.

inhibitory effects against sucrase activity (Table 3.5), with that of *Q. phillyraeoides* showing the greatest effect (90%).

The hexane and water extracts of five plant species showed weak inhibitory effects on rat intestinal sucrase activity (Table 3.5). The ethyl acetate and butanol extracts of *Q. phillyraeoides* were shown to inhibit the enzyme activity by 63% and 57%, respectively (Table 3.5). However, the hexane and water extracts of *Q. phillyraeoides* leaves showed low levels of inhibition on sucrase activity, while acarbose (positive control) showed 91% inhibition of the enzyme activity at a concentration of 1 mg/ml (Table 3.5).

The ethyl acetate and butanol extracts of *M. japonicus* leaves showed the greatest inhibitory effects (57% and 67%, respectively), and the weakest effects were observed for hexane and water extracts of *M. japonicus* (35% and 50%, respectively).

The ethyl acetate and butanol extracts of *E. sylvestris* var. *ellipticus* leaves showed the greatest inhibitory effects (70% and 62%, respectively) against sucrase activity (Table 3.5). The butanol extract of *M. rubra* leaves showed an inhibitory effect of 48%, while the hexane, ethyl acetate, and water extracts showed lower inhibitory activities of 30%, 41%, and 28%, respectively (Table 3.5). The hexane, ethyl acetate, butanol, and water extracts of *S. sebiferum* showed high inhibitory activities of 51%, 63%, 76%, and 56%, respectively (Table 3.5).

3.4 Discussion

In this study, we used acarbose as a positive control for both yeast α-glucosidase and rat intestinal α-glucosidase. Acarbose did not show a stronger inhibitory effect against yeast α-glucosidase, whereas it showed the greatest inhibitory effect against rat intestinal α-glucosidase. The differences in the inhibitory activities against these two enzymes may have been due to their structural differences (Chiba, 1997). It was confirmed that the experimental protocols were identical because acarbose was used as a positive control for both enzymes. It is important to note that acarbose has been used clinically to treat diabetes mellitus. Therefore, several groups have used acarbose as a positive control to identify or screen for suitable natural antidiabetic compounds (Zhang *et al*., 2014; Ghadyale *et al*., 2011; Wu *et al*., 2011).

Although acarbose has been clinically approved for use as an antidiabetic medication, Shai *et al*. (2010) reported that acarbose showed a weak inhibitory effect against yeast α-glucosidase. This result was compatible with the present study, in which acarbose showed only a weak inhibitory effect against yeast α-glucosidase with an IC_{50} value of 13 mg/ml, while the five plant extracts showed strong inhibitory effects against the activity of this enzyme (Table 3.4).

The methanol, ethyl acetate, and butanol extracts of the leaves from the five plant species, *Q. phillyraeoides*, *M. japonicus*, *S. sebiferum*, *E. sylvestris* var. *ellipticus*, and *M. rubra*, showed stronger inhibitory effects against yeast α-glucosidase than rat intestinal sucrase (Supplemental Data, Table 3.5). At 200 μg/ml, methanol, ethyl acetate, and butanol extracts of *Q. phillyraeoides* inhibited yeast α-glucosidase activity by 99%, 100%, and 99%, respectively, while at 1 mg/ml, the inhibitory effects of these extracts on rat intestinal sucrase were lower at 90%, 63%, and 57%, respectively. This result was similar by those of Babu *et al*. (2004), who reported that methanol extracts of various plants had greater inhibitory effects against yeast α-glucosidase than mammalian α-glucosidase.

The ethyl acetate and butanol fractions of *Q. phillyraeoides* had IC_{50} values for yeast α-glucosidase of 4 μg/ml and 6 μg/ml, respectively (Table 3.4). Dewi *et al*. (2007) reported that the ethyl acetate extract of *koji Aspergillus terreus* exhibited a strong inhibitory effect against α-glucosidase with an IC_{50} value of 8.6 μg/ml. These findings

indicated that α-glucosidase inhibitory activity of *Q. phillyraeoides* was stronger than that of *koji Aspergillus terreus*, which is known as a potent α-glucosidase inhibitor.

These results of the present study indicated that the methanol extract of *Q. phillyraeoides* leaves showed the strongest inhibitory effect against rat intestinal sucrase activity (90%), followed by those of *E. sylvestris* var. *ellipticus* (77%), *M. rubra* (76%), *M. japonicus* (73%), and *S. sebiferum* (69%) (Supplemental Data, Table 3.5). On the other hand, Kajaria *et al*. (2013) reported that ethanolic extract of *Shirishadi* showed a strong inhibitory effect of 45% (1 mg/ml) against α-glucosidase. The results suggested that α-glucosidase inhibitory activity of *Q. phillyraeoides* was higher than that of ethanolic extract of *Shirishadi*, which has been identified as a potent α-glucosidase inhibitor.

3.5 Conclusions

The results of the present study indicated that different extracts of five plants (*E. sylvestris* var. *ellipticus*, *M. japonicus*, *M. rubra*, *Q. phillyraeoides*, and *S. sebiferum*) showed strong inhibitory effects against yeast α-glucosidase activities. *Q. phillyraeoides* (ethyl acetate) leaf extract showed the greatest inhibitory effect against yeast α-glucosidase. Methanol, ethyl acetate, and butanol extracts of *E. sylvestris* var. *ellipticus*, *M. japonicus*, *M. rubra*, *Q. phillyraeoides*, and *S. sebiferum* showed strong inhibitory effects against sucrase activity. The leaves of these five plant species should be investigated further to purify and identify the specific compound(s) responsible for the observed potential antidiabetic activities.

Chapter 4

Isolation and purification of α-glucosidase inhibitory constituents from *Terminalia chebula*, *Mallotus japonicus*, and *Quercus phillyraeoides*

Abstract

α-Glucosidase inhibitors can delay the carbohydrate digestion process in the small intestine and control postprandial blood glucose level. In this study, butanol extract of *Terminalia chebula*, butanol extract of *Mallotus japonicus*, and ethyl acetate extract of *Quercus phillyraeoides* were screened for biologically active compounds using a fractionation technique and yeast α-glucosidase inhibitory activity assay. Two fractions (TCB-1 and TCB-2) were isolated from the leaves of *T. chebula*, one fraction (MJB-1) was isolated from the leaves of *M. japonicus*, and three fractions (QPE 1, QPE-2, and QPE-3) were isolated from the leaves of *Q. phillyraeoides*. The half maximal concentrations (IC_{50}) of TCB-1, TCB-2, and MJB-1 fractions were 2 μg/ml, 3 μg/ml, and 10 μg/ml, respectively, and those of QPE 1, QPE-2, and QPE-3 fractions were 1.6 μg/ml, 26 μg/ml, and 27 μg/ml, respectively. All six fractions showed significant *in vitro* α-glucosidase inhibitory activity compared with acarbose (IC_{50} value: 13 mg/ml). These results suggest that the three plants may be useful as sources of natural α-glucosidase inhibitors for use in the treatment of diabetes.

Keywords: α-glucosidase inhibitor, diabetes, *Terminalia chebula*, *Mallotus japonicus*, *Quercus phillyraeoides*

4.1 Introduction

We screened for α-glucosidase inhibitors from the leaf extracts of 22 plant species from Bangladesh and Japan to use in the treatment of diabetes (Chapters 2 and 3). The dry leaves were extracted with methanol and then dried using a rotary evaporator. The dry extract was partitioned in 30% hexane/70% methanol solution, followed by fractionation with ethyl acetate, butanol, and water. Each fraction was evaporated under reduced pressure to provide hexane, ethyl acetate, butanol, and water fractions. Different extracts of eight plant species showed significant inhibitory effects against yeast α-glucosidase.

As discussed in Chapter 2, the leaf extracts of three Bangladeshi medicinal plants *T. chebula*, *T. bellirica*, and *E. officinalis* showed significant inhibitory effects against yeast α-glucosidase. The main purpose of this study was to find new traditional medicinal plants for diabetes among Bangladeshi plants, but the butanol extract of *T. chebula* was selected for fractionation because it showed high inhibitory effect against yeast α-glucosidase.

Chapter 3 discussed the screening of leaf extracts from 19 plant species for yeast α-glucosidase inhibitory activities. Among them, different extracts of five plants (*Elaeocarpus sylvestris var. ellipticus*, *Mallotus japonicus*, *Myrica rubra*, *Quercus phillyraeoides*, and *Sapium sebiferum*) showed strong inhibitory effects against yeast α-glucosidase. Among these plants, ethyl acetate extract of *Q. phillyraeoides* and butanol extract of *M. japonicus* showed the strongest inhibitory effects against yeast α-glycosidase. The main purpose of this study was to find new compounds for treatment of diabetes from plant leaves collected in Japan.

Further chemical investigation of the butanol extract of *T. chebula*, butanol extract of *M. japonicus*, and ethyl acetate extract of *Q. phillyraeoides* are necessary to develop new pharmaceutical preparations. Therefore, these three plants were screened for biologically active compounds using a fractionation technique and yeast α-glucosidase inhibitory activity assay.

Many biologically active compounds have been discovered from plants and reported to show antidiabetic effects. Alkaloids, terpenoids, and their derivates have been used for their anti-hyperglycemic activity from ancient times (Erememisoglu *et al*., 1995).

Flavonoids are polyphenolic compounds that play important roles in reducing blood glucose level (Babu *et al*., 2013).

In the present study, a fractionation technique was used for isolation and purification of yeast α-glucosidase inhibitors from plant extracts. Silica gel column chromatography (Si-gel CC), medium-pressure liquid chromatography (MPLC), and high-performance liquid chromatography (HPLC) were used to separate and purify different components from mixtures of plant extracts. The collected fractions were examined by thin layer chromatography (TLC) for desired components.

4.2 Materials and methods

4.2.1 Plant materials

The butanol extract of *T. chebula* (Chapter 2), butanol extract of *M. japonicas* (Chapter 3), and ethyl acetate extract of *Q. phillyraeoides* (Chapter 3) fractions were used for isolation and purification.

4.2.2 Isolation and purification of α-glucosidase inhibitors from *T. chebula*

4.2.2.1 Silica gel column chromatography (Si-gel CC)

Silica gel column chromatography was used to separate components from the active fraction. An open glass column (54cm × 4cm id.; Vidrex, Fukuoka, Japan) and silica gel (FL100D; Fuji Silysia, Kasugai, Japan) were used for column chromatography. Cotton was added to the bottom of the column to prevent the silica from being washed out. For column preparation, dry silica gel was suspended in 100% ethyl acetate and poured into the column. The stopcock was opened for flowing out ethyl acetate up to an equal level with silica gel. After 5 minutes, the sample was poured into the silica gel column. For sample preparation, a small amount of 50% methanol was mixed with the sample. Completely soluble samples were loaded directly onto the silica gel column. The sample was eluted with solvent mixtures and fractions of 50 ml were collected. If the sample was not soluble in methanol, about 1 ml of insoluble sample was poured into the silica gel-containing flask and evaporated until the entire sample had been used. The completely evaporated sample was dried by lyophilization.

The butanol extract of *T. chebula* (394 mg) was mixed with 50% methanol and loaded onto the column followed by elution with a step gradient procedure, which gradually increases in polarity. In the first step, the sample was eluted with ethyl acetate:methanol:water (16:2:1, 950 ml v/v/v) to yield fractions 1 – 19. In the second step, the sample was eluted with ethyl acetate:methanol:water (8:2:1, 1050 ml v/v/v) to yield fractions 20 – 40. In the third step, the sample was eluted with ethyl acetate:methanol:water (4:2:1, 500 ml v/v/v) to yield fractions 41 – 53 and finally washed with methanol (500 ml).

4.2.2.2 High-performance liquid chromatography (HPLC)

HPLC was used for separation of different compounds from the active fractions based on their polarity and hydrophobic interactions. The HPLC system consisted of a

pump (PU-2089 plus; Jasco, Tokyo, Japan), column oven (CO-2065 plus; Jasco), and detector (UV-2077; Jasco). A stainless steel column was used in this experiment, and the column oven was set at 40°C. An octadecyl silyl (ODS) column was used for separation of active fractions. The UV detector was used to monitor fractions and compounds eluted from the column. The ODS column is recommended for reverse-phase chromatography in which a polar mobile phase and a non-polar stationary phase are used. This allows non-polar molecules (hydrophobic) coupled to carrier beads (silica C18) and polar molecules (hydrophilic) to pass swiftly through the stationary phase. Polar solvents (methanol and water) were used to elute the binding molecules in this study. The sample was dissolved in a small amount of methanol and fractions were collected by peak assignment.

For final purification, *T. chebula* extract (131 mg, B1 fraction) was subjected to HPLC under the following conditions: column, YMC pack ODS-A A-301 100 × 4.6 mm I. D. with a particle size of 5 µm and pore size of 120 Å; isocratic elution, 25% methanol; flow rate, 1.5 ml/min; detection, UV 210 nm; and chart speed, 15 cm/h. The injection volume was 20 µl.

4.2.2.3 Thin layer chromatography (TLC)

All chromatographic fractions were checked by thin layer chromatography (TLC) and the fractions that showed similar spots were combined for yeast α-glucosidase assay. TLC was performed on precoated plates (Silica gel 60 F_{254}; Merck, Tokyo, Japan; RP-18 WF_{254s}; Merck, Darmstadt, Germany). The chromatographic fractions were spotted onto the TLC plate. The developing tank was prepared with a suitable solvent. The TLC plate was then dipped into the developing tank for a few minutes until the solvent ascended to the top of the plate. The TLC plate was visualized under UV light and developed by spraying with ethanol: 20% H_2SO_4 (1:1) and heating at 110°C to identify the presence of antidiabetic compounds.

4.2.3 Isolation and purification of α-glucosidase inhibitors from *M. japonicus*

4.2.3.1 Silica gel column chromatography (Si-gel CC)

The column and sample preparation process were as described above for Si-gel CC of *T. chebula* (Subsection 4.2.2.1) but the sample volume, number of fractions, and proportions of solvents were different, as outlined below.

The butanol extract of *M. japonicus* (2.5 g) was mixed with 50% methanol and loaded onto the column. In the first step, the sample was eluted with ethyl acetate:methanol (4:1, 650 ml v/v) to yield fractions 1 – 13. In the second step, the sample was eluted with ethyl acetate:methanol:water (16:2:1, 650 ml v/v/v) to yield fractions 14 – 26. In the third step, the sample was eluted with ethyl acetate:methanol:water (6:2:1, 450 ml v/v/v) to yield fractions 27 – 36 and finally washed with methanol (500 ml).

4.2.3.2 Medium-pressure liquid chromatography (MPLC)

Medium-pressure liquid chromatography (MPLC) was used to separate components from the active fraction. The active fraction was subjected to MPLC using a glass column (YMC-GEL ODS-A, 15 cm × 16 mm I.D.) with a particle size of 150 µm and pore size of 120 Å. The sample dissolved in a small amount of methanol, and the dissolved sample was loaded onto the column. The fractions were collected using a fraction collector (SF-3120; Advantec, Ehime, Japan).

The butanol fraction of *M. japonicus* extract (2015 mg, B2 fraction) was separated by MPLC with the following elution conditions: elution gradient, 0% – 100% methanol; and flow rate, 1.5 ml/min. The samples were run for 15 hours, and the volume of each fraction was 15 ml.

4.2.3.3 High-performance liquid chromatography (HPLC)

The high-performance column chromatography (HPLC) system (pump, oven, and detector), ODS column, and sample preparation were as described for *T. chebula* (Subsection 4.2.2.2), but with the sample volume, column conditions, and injection volume outlined below.

For final purification, *M. japonicus* extract (247 mg, B2-4 fraction) was subjected to HPLC with the following conditions: column, YMC pack ODS-5-ST 150 × 20 mm I. D. with a particle size of 5 µm and pore size of 120 Å; isocratic elution, solvent 50% methanol; flow rate, 5 ml/min; detection, UV 210 nm; and chart speed, 15 cm/h. The injection volume was 50 µl.

4.2.3.4 Thin layer chromatography (TLC)

All chromatographic fractions were checked by thin layer chromatography (TLC) according to the procedure described for *T. chebula* (Subsection 4.2.2.3).

4.2.4 Isolation and purification of α-glucosidase inhibitors from *Q. phillyraeoides*

4.2.4.1 Silica gel column chromatography (Si-gel CC)

The column and sample preparation process were as described for Si-gel CC of *T. chebula* (Subsection 4.2.2.1) but with the sample volume, number of fractions, and proportion of solvents outlined below.

The ethyl acetate extract of *Q. phillyraeoides* (368 mg, E1-1 fraction) was mixed with 50% methanol and loaded onto the column. The column was eluted with an isocratic system. A range of ratios was used for the column: ethyl acetate:methanol:water, 8:2:1, 950 ml v/v/v to yield 19 fractions.

4.2.4.2 Medium-pressure liquid chromatography (MPLC)

The column, fraction collector, and sample preparation process were as described for MPLC of *M. japonicus* (Subsection 4.2.3.2), but with the sample volume, elution conditions, sample run time, and fraction volumes outlined below.

The ethyl acetate extract of *Q. phillyraeoides* (2 g) was separated by MPLC with the following elution conditions: gradient elution, 50% – 100% methanol; and flow rate, 2.5 ml/min. The samples were run for 9 hours, and the volume of each fraction was 11.25 ml.

The ethyl acetate extract of *Q. phillyraeoides* (910 mg, E1 fraction) was separated by MPLC with the following elution conditions: gradient elution, 80% – 100% methanol; and flow rate, 2.0 ml/min. The samples were run for 10 hours, and the volume of each fraction was 9.8 ml.

4.2.4.3 High-performance liquid chromatography (HPLC)

The HPLC system (pump, oven, and detector), ODS column, and sample preparation process were as described for HPLC of *T. chebula* (Subsection 4.2.2.2), but with the sample volume, column conditions, and injection volume outlined below.

For final purification, *Q. phillyraeoides* extract (197 mg, E1-1-1 fraction) was subjected to HPLC under the following conditions: column, YMC pack ODS-A A-301 100 × 4.6 mm I. D. with a particle size of 5 μm and pore size of 120 Å; isocratic elution, 35% methanol; flow rate, 1.0 ml/min; detection, UV 210 nm; and chart speed, 15 cm/h. The injection volume was 20 μl.

For final purification, *Q. phillyraeoides* extract (30 mg, E2 fraction) was subjected to HPLC under the following conditions: column, YMC pack ODS-5-ST 150 × 20 mm I. D. with a particle size of 5 μm and pore size of 120 Å; isocratic elution, 60% methanol (0.05% trifluoroacetic acid); flow rate, 5.0 ml/min; detection, UV 210 nm; and chart speed, 15 cm/h. The injection volume was 50 μl.

Q. phillyraeoides extract (130 mg, E4 fraction) was subjected to HPLC with the following conditions: column, YMC pack ODS-5-ST 150 × 20 mm I. D. with a particle size of 5 μm and pore size of 120 Å; isocratic elution, 60% methanol (0.05% trifluoroacetic acid); flow rate, 5.0 ml/min; detection, UV 210 nm; and chart speed, 15 cm/h. The injection volume was 50 μl.

For final purification, *Q. phillyraeoides* extract (10 mg, E4-2 fraction) was subjected to HPLC with the following conditions: column, YMC pack ODS-5-ST 100 × 4 mm I. D. with a particle size of 5 μm and pore size of 120 Å; isocratic elution, 50% methanol; flow rate, 1.0 ml/min; detection, UV 210 nm; and chart speed, 15 cm/h. The injection volume was 20 μl.

4.2.4.4 Thin layer chromatography (TLC)

All chromatographic fractions were checked by thin layer chromatography (TLC) according to the procedure described for *T. chebula* (Subsection 4.2.2.3).

4.2.5 Yeast α-glucosidase inhibitory assay

The yeast α-glucosidase inhibitory activity assay was performed as described in Chapter 2.

4.3 Results

4.3.1 *T. chebula*

The butanol extract was used as the starting material for separation of compounds with inhibitory activities of α-glucosidase in *T. chebula* (Fig. 4.1). The butanol extract of *T. chebula* was separated by silica gel column chromatography (step gradient) with varying concentrations of the solvent, ethyl acetate:methanol:water (16:2:1, 8:2:1, 4:2:1), yielding 53 fractions and finally the sample was washed with methanol, yielding 54 number of fraction. Each fraction was checked by TLC and according to the spot pattern, seven combined fractions were obtained as follows: B1 (Fr.1 – Fr.4), B2 (Fr.5 – Fr.9), B3 (Fr.10 – Fr.21), B4 (54, methanol fraction), B5 (Fr.22 – Fr.34), B6 (Fr.35 – Fr.40), and B7 (Fr.41 – Fr.53) (data not shown). Four fractions were checked by

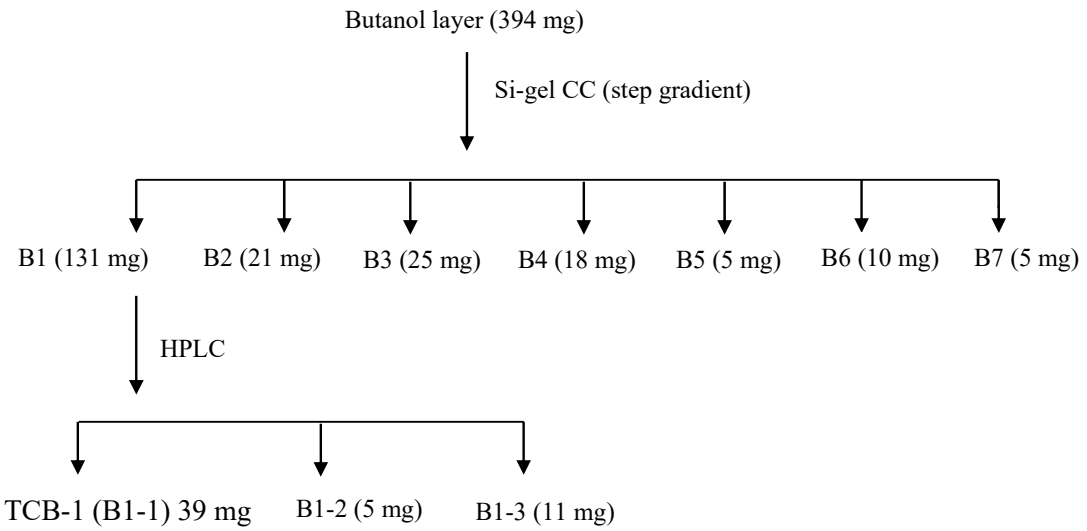

Fig. 4.1 Scheme of the separation of fractions from *Terminalia chebula* butanol extract.

Table 4.1 Inhibitory effects of B1 – B7 fractions from *Terminalia chebula* butanol extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
B1	6
B2	14
B3	6
B4	7
B5	7
B6	9
B7	34

Fig. 4.2 Thin layer chromatography of B1 – B4 fractions of *T. chebula* butanol extract. Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of ethyl acetate : methanol : water (8:2:1). Spots appeared in fraction B1.

TLC (Fig. 4.2). Among them, spots appeared in the B1 fraction. These fractions showed α-glucosidase inhibitory activities with IC_{50} values of 6 μg/ml (B1), 14 μg/ml (B2), 6 μg/ml (B3), 7 μg/ml (B4), 7 μg/ml (B5), 9 μg/ml (B6), and 34 μg/ml (B7) (Table 4.1). Among those fractions, the B1 fraction showed the greatest inhibitory effect with an IC_{50} value of 6 μg/ml. Thereafter, the B1 fraction was purified by HPLC to yield B1-1 (I), B1-2 (II), and B1-3 (remaining part, III) fractions. As shown in Fig. 4.3, fractions were

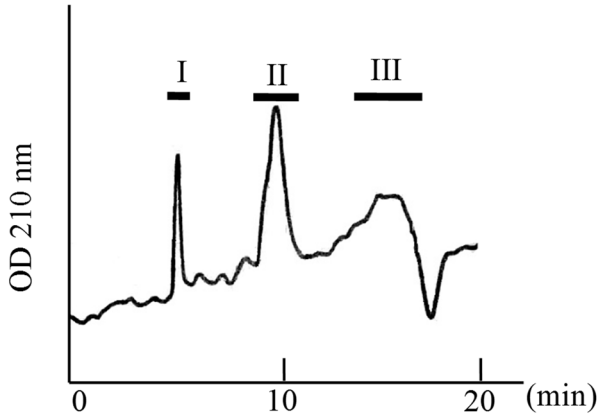

Fig. 4.3 High-performance liquid chromatography (HPLC) of B1 fraction of *T. chebula* butanol extract. The peaks were found at retention times of 6 (I, B1-1) and 10 (II, B2-4-2) minutes by UV monitoring at 210 nm.

Table 4.2 Inhibitory effects of B1-1, B1-2, and B1-3 fractions from B1 fraction of *Terminalia chebula* butanol extract against yeast α-glucosidase.

Fraction	IC_{50} μg/ml
B1-1 (TCB-1)	2
B1-2 (TCB-2)	3
B1-3	20

collected at retention times of 6 (I) and 10 (II) minutes by UV monitoring at 210 nm. The collected fractions showed α-glucosidase inhibitory activities with IC_{50} values of 2 μg/ml (B1-1), 3 μg/ml (B1-2), and 20 μg/ml (B1-3) (Table 4.2). Among these fractions, B1-1 (TCB-1) and B1-2 (TCB-2) fractions showed strong inhibitory effects against yeast α-glucosidase (Table 4.2).

4.3.2 *M. japonicus*

The butanol extract was used as the starting material for separation of compounds with inhibitory activities against α-glucosidase in *M. japonicus* (Fig. 4.4). The butanol extract of *M. japonicus* was fractionated by silica gel column chromatography (step gradient) and eluted with varying concentrations of solvent ethyl acetate:methanol (4:1), ethyl acetate:methanol:water (16:2:1), ethyl acetate:methanol:water (6:2:1). Finally, 37 fractions were collected by silica gel column chromatography. Each fraction was checked by TLC and according to spot pattern, seven

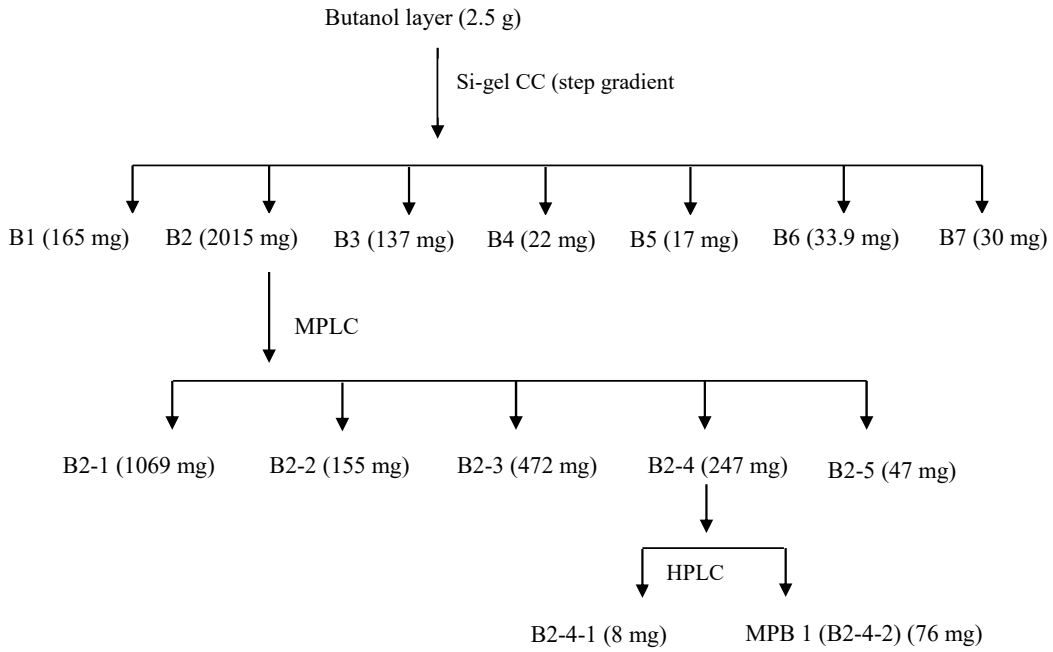

Fig. 4.4 Scheme of the separation of fractions from *Mallotus japonicus* butanol extracts.

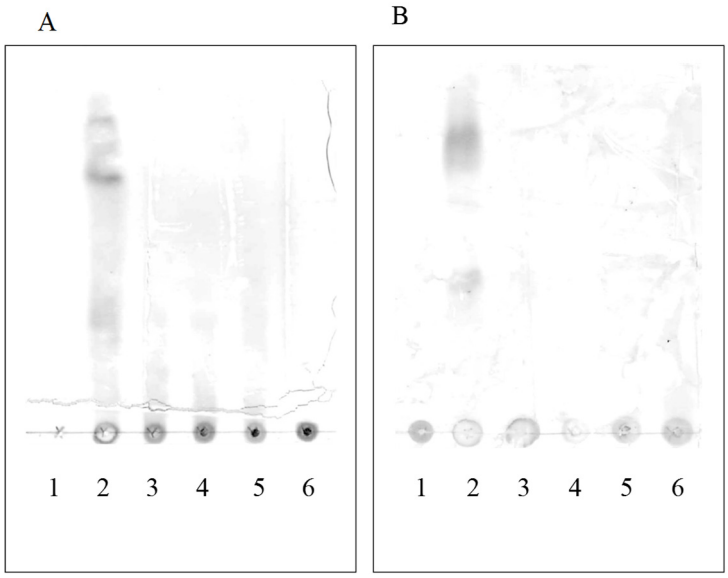

Fig. 4.5 Thin layer chromatography (TLC) of B1 – B6 fractions of *M. japonicus* butanol extract.

A: Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of ethyl acetate:methanol:water (6:2:1). The spots appeared in fraction B2.

B: Chromatographic fractions were checked by reverse-phase thin layer chromatography (RP-18 F_{254}s) using 50% methanol. The spots also appeared in fraction B2.

combined fractions were obtained as follows: B1 (Fr.1 – Fr.2), B2 (Fr.3 – Fr.5), B3 (Fr.6 – Fr.13), B4 (Fr.14 – Fr.18), B5 (Fr.19 – Fr.30), B6 (Fr.31 – Fr.36), and B7 (37) (data not shown). Six fractions were then checked by TLC using the solvent, ethyl acetate:methanol:water (6:2:1, 9 ml v/v/v) (Fig. 4.5A) and 50% methanol (Fig. 4.5B). The spots appeared in the B2 fraction on both TLC plates. These fractions showed α-glucosidase inhibitory effects, with IC_{50} values of 28 µg/ml (B1), 11 µg/ml (B2), 40 µg/ml (B3), 33 µg/ml (B4), 33 µg/ml (B5), and 38 µg/ml (B6); the B7 fraction showed no inhibitory activity (Table 4.3). Among these fractions, B2 fraction showed the highest

Table 4.3 Inhibitory effects of B1 – B7 fractions from *Mallotus japonicus* butanol extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
B1	28
B2	11
B3	40
B4	33
B5	33
B6	38
B7	NA

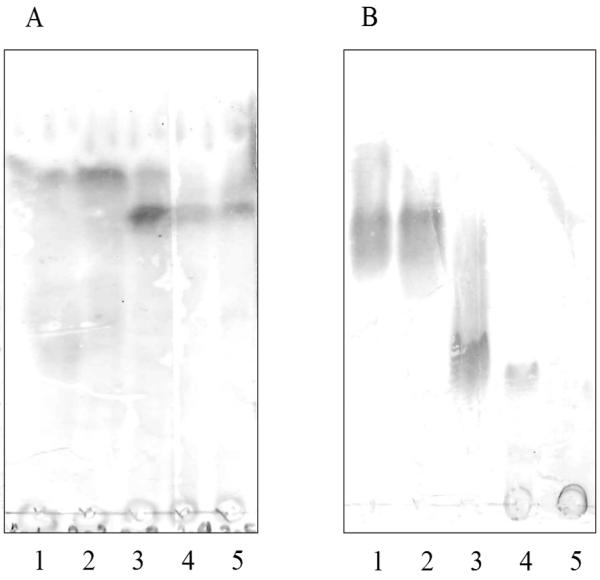

Fig. 4.6 Thin layer chromatography of B2-1 – B2-5 fractions of *M. japonicus* butanol extract.

A: Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of ethyl acetate:methanol:water (6:2:1). Spots were not clear in B2-1 – B2-5 fractions.

B: Chromatographic fractions were checked by reverse-phase thin layer chromatography (RP-18 F_{254}s) using 50% methanol. B2-1 – B2-3 fractions showed smeared spot, B2-4 fraction showed a clear spot. B2-5 fraction did not show any spots

inhibitory effect with an IC_{50} value of 11 µg/ml (Table 4.3). The B2 fraction was then fragmented by MPLC and yielded 120 fractions. Chromatographic fractions were examined by TLC (data not shown). According to the spot pattern, five combined fractions were obtained as follows: B2-1 (1 – 29), B2-2 (30 – 44), B2-3 (45 – 65), B2-4 (66 – 105), and B2-5 (106 – 120) (data not shown). The B2-1 – B2-5 fractions were then checked by TLC (Fig. 4.6). Among these fractions, spots appeared in the B2-4 fraction. The B2-1 – B2-5 fractions were checked for α-glucosidase inhibitory activities, and showed IC_{50} values of 16 µg/ml (B2-1), 17 µg/ml (B2-2), 16 µg/ml (B2-3), 10 µg/ml (B2-

Table 4.4 Inhibitory effects of B1 – B7 fractions from *Mallotus japonicus* butanol extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
B2-1	16
B2-2	17
B2-3	16
B2-4	10
B2-5	48

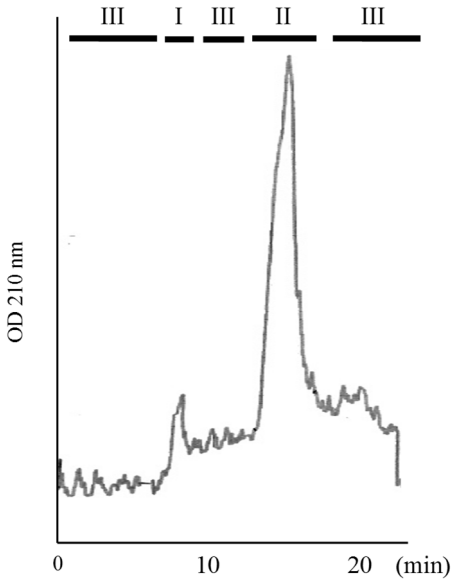

Fig. 4.7 High-performance liquid chromatography (HPLC) of B2-4 fraction of *M. japonicus* butanol extract. The peaks were found at retention times of 8 (I, B2-4-1) and 15 (II, B2-4-2) minutes by UV monitoring at 210 nm.

4), and 48 µg/ml (B2-5) (Table 4.4). Among these fractions, the B2-4 fraction showed maximum inhibitory activity. According to the spot and inhibitory activity, the B2-4 fraction was selected for further fractionation, and HPLC yielded B2-4-1 (I), B2-4-2 (II), and B2-4-3 (remaining part, III) fractions (Fig. 4.7). The fractions were collected at retention times of 8 (I) and 15 (II) minutes by UV monitoring at 210 nm. The B2-4-2 (II) fraction showed an individual peak (Fig. 4.7). The fractions were then checked by TLC;

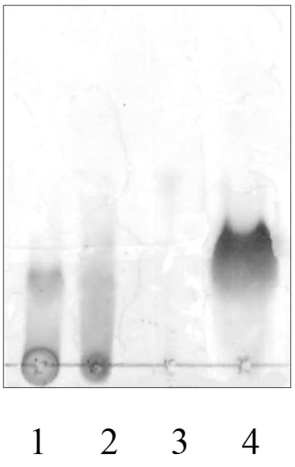

Fig. 4.8 Thin layer chromatography (TLC) of B2-4-1 and B2-4-2 fraction of *M. japonicus* butanol extract. Chromatographic fractions were checked by reverse-phase thin layer chromatography (RP-18 F_{254}s) using 50% methanol. Lane 1 (B2-4 main) showed a clear spot and lane 2 (B2-4-3) showed a smeared spot, lane 3 (B2-4-1) showed no spot, and lane 4 (B2-4-2) showed a clear spot.

Table 4.5 Inhibitory effects of B2-4-1 and B2-4-2 fractions from B2-4 fraction of *Mallotus japonicus* butanol extract against yeast α-glucosidase.

Fraction	IC_{50} μg/ml
B2-4-1	17
B2-4-2 (BJB-1)	10

lane 1 (B2-4) showed a clear spot, lane 2 (B2-4-3) showed a smeared spot, B2-4-1 showed no spot, and B2-4-2 fraction showed a clear spot (Fig. 4.8). These fractions were examined for α-glucosidase inhibitory activities, and showed IC_{50} values of 17 μg/ml (B2-4-1) and 10 μg/ml (B2-4-2) (Table 4.5). The B2-4-2 fraction showed the highest inhibitory effect against yeast α-glucosidase.

4.3.3 *Q. phillyraeoides*

The ethyl acetate extract was used as the starting material for separation of compounds with inhibitory activities against α-glucosidase in *Q. phillyraeoides* (Fig. 4.9).

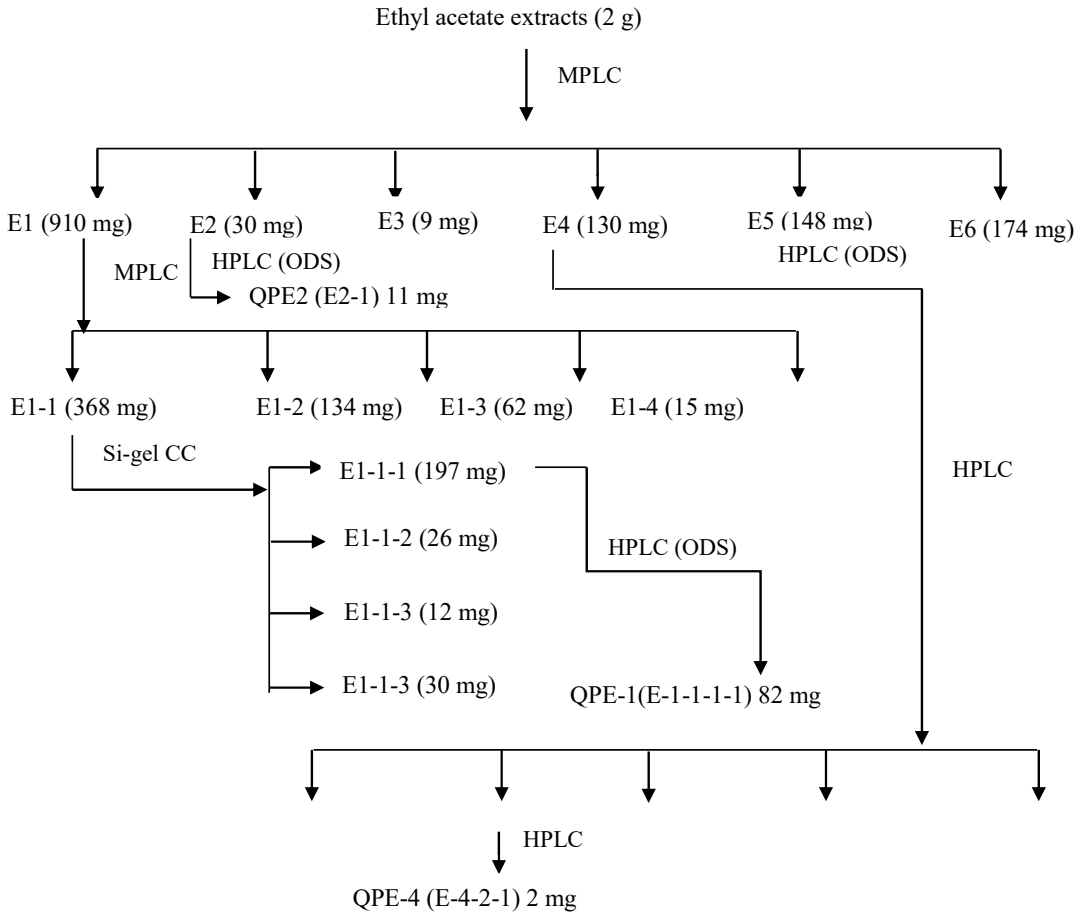

Fig. 4.9 Scheme of the separation of fractions from *Quercus phillyraeoides* ethyl acetate extract.

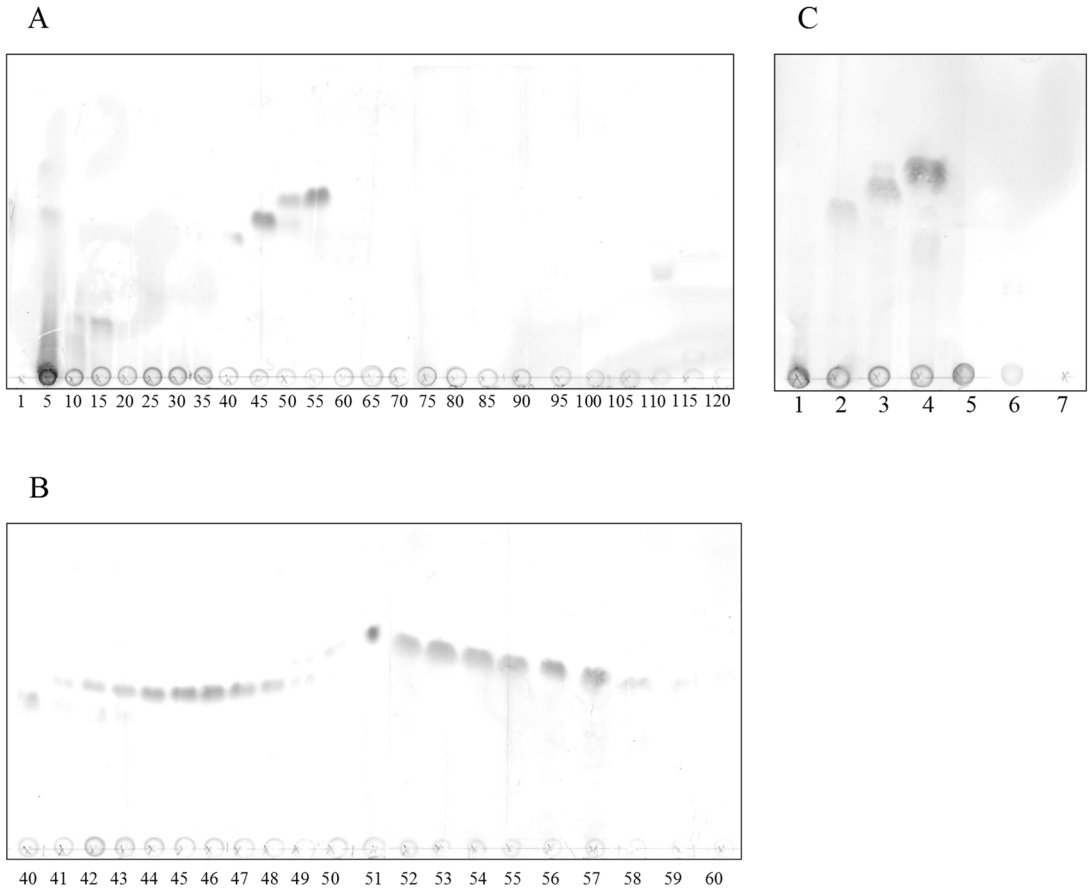

Fig. 4.10 Thin layer chromatography (TLC) of *Q. phillyraeoides* ethyl acetate extract.

A: Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of hexane:ethyl acetate:methanol (5:5:2). Based on the lane spot pattern, fractions 1 – 34 were combined in to the E1 fraction, fractions 35 – 40 were combined into the E2 fraction, fractions 61 – 100 were combined into the E5 fraction, and fractions 101 – 120 were combined into the E6 fraction.

B: Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of hexane:ethyl acetate:methanol (5:5:2). Fractions 41 – 50 were combined into the E3 fraction and fractions 51 – 60 were combined into the E4 fraction

C: Thin layer chromatography (TLC) of E1 – E6 fractions of *Q. phillyraeoides* ethyl acetate extract. Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of hexane:ethyl acetate:methanol (5:5:2). The results for fractions E1 – E6 are shown. Among the fractions, E1 – E4 (Lanes 1 – 4) fractions showed clear spots and E5 – E6 (Lanes 5 – 6) fractions did not show any spots

The ethyl acetate extract of *Q. phillyraeoides* was separated by MPLC (120 tubes) and collected fractions were checked by TLC (Fig. 4.10A). Lanes 40 – 60 showed clear spots with different mobilities, so it was difficult to distinguish between these lanes. According to spot appearing condition, it was checked more details by TLC (Fig. 4.10B). Based on

Table 4.6 Inhibitory effects of E1 – E5 fractions of *Quercus phillyraeoides* ethyl acetate extract against yeast α-glucosidase.

Fraction	IC_{50} μg/ml
E1	3
E2	14
E3	16
E4	35
E5	NA
E6	NA

the spot patterns, as shown in Figs. 4.10A and 4.10B, six combined fractions were obtained as follows: E1 (Fr.1 – Fr.34), E2 (Fr.35 – Fr.40), E3 (Fr.41 – Fr.50), E4 (Fr.51 – Fr.60), E5 (Fr.61 – Fr.100), and E6 (Fr.101 – Fr.120). Figure 4.10C shows the final TLC patterns of E1 – E6. Among these, E1 – E4 fractions showed clear spots and E5 – E6 fractions did not show any spots. These fractions were examined for α-glucosidase inhibitory activities, and showed IC_{50} values of 3 μg/ml (E1), 14 μg/ml (E2), 16 μg/ml (B3), and 35 μg/ml (E4) (Table 4.6). E5 and E6 fractions showed no inhibitory activity (Table 4.6). The fractions E1 – E4 showed excellent inhibitory effects on α-glucosidase activity, with E1 showing the strongest inhibitory effect (IC_{50}: 3 μg/ml).

Finally, ethyl acetate extract of *Q. phillyraeoides* (2000 g) was combined into six fractions with weights of 910 mg (E1), 30 mg (E2), 9 mg (E3), 130 mg (E4), 148 mg (E5), and 174 mg (E6) (Fig. 4.9). Three active fractions (E1, E2, and E4) were examined to purify the active compounds for inhibition against α-glucosidase according to the method described in Fig. 4.9. Although E3 fraction (9 mg) also showed high inhibitory activity, the amount of extract was not sufficient for further purification.

E1 fraction was fragmented by MPLC (120 fractions). For primary investigation, every five consecutive fractions were checked from 120 fractions (1-5-10-15------120, this way). According to the spot pattern, 120 fractions were combined as follows: E1-1 (Fr.1 – Fr.55), E1-2 (Fr.56 – Fr.74), E1-3 (Fr.75 – Fr.90), E1-4 (Fr.91 – Fr.104), and E1-5 (Fr.105 – Fr.120) (data not shown). The E1-1 – E1-5 fractions were checked by TLC using a mixture of hexane:ethyl acetate:methanol (5:5:2) (Fig. 4.11A) and ethyl acetate:methanol (5:1) (Fig. 4.11B). Among the fractions, E1-1 fraction showed a large spot, but the spot did not move. Therefore, it was not possible to determine whether this spot contained one or more molecules. E1-2 and E1-3 fractions showed spots but their patterns were not clear. E1-4 and E1-5 fractions did not show any spots (Fig. 4.11A).

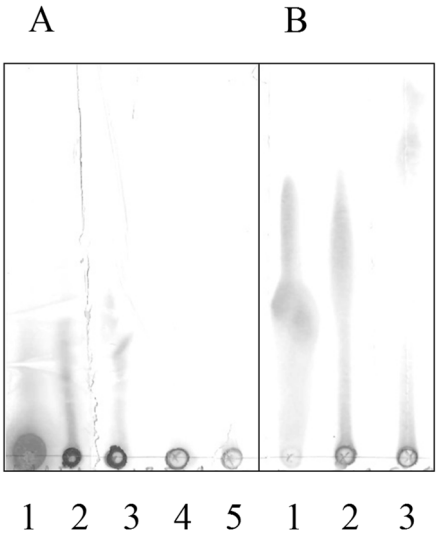

Fig. 4.11 Thin layer chromatography (TLC) of E1-1 – E1-5 fractions of *Q. phillyraeoides* ethyl acetate extract.

A: Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of hexane:ethyl acetate:methanol (5:5:2). Among the fractions, E1-1 fraction showed a large spot. E1-2 (Lane 2) and E1-3 (Lane 3) showed smeared spots, and E1-4 (Lane 4) and E1-5 (Lane 5) fractions did not show any spots.

B: Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of ethyl acetate:methanol (5:1). The E1-1(Lane 1) spot was dark black, while E1-2 (Lane 2) and E1-3 (Lane 3) fractions showed smeared spots.

The E1-1 fraction showed a dark black spot, which moved but did not separate. Therefore, it was not possible to determine whether this spot contained one or more molecules (Fig. 4.11B). E1-2 and E1-3 fractions showed smears (Fig. 4.11B). These fractions were examined for α-glucosidase inhibitory activities, and showed IC_{50} values of 3 µg/ml (E1-1), 7 µg/ml (E1-2), 9 µg/ml (E1-3), 9 µg/ml (E1-4), and 11 µg/ml (E1-5) (Table 4.7). E1-1 fraction showed the strongest inhibitory effect against yeast α-glucosidase with an IC_{50} value of 3 µg/ml. The E1-1 fraction was again subjected to silica gel column chromatography, and each resulting fraction was checked by TLC. According to the spot

Table 4.7 Inhibitory effects of E1-1 – E1-5 fractions from E1 fraction of *Quercus phillyraeoides* ethyl acetate extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
E1-1	3
E1-2	7
E1-3	9
E1-4	9
E1-5	11

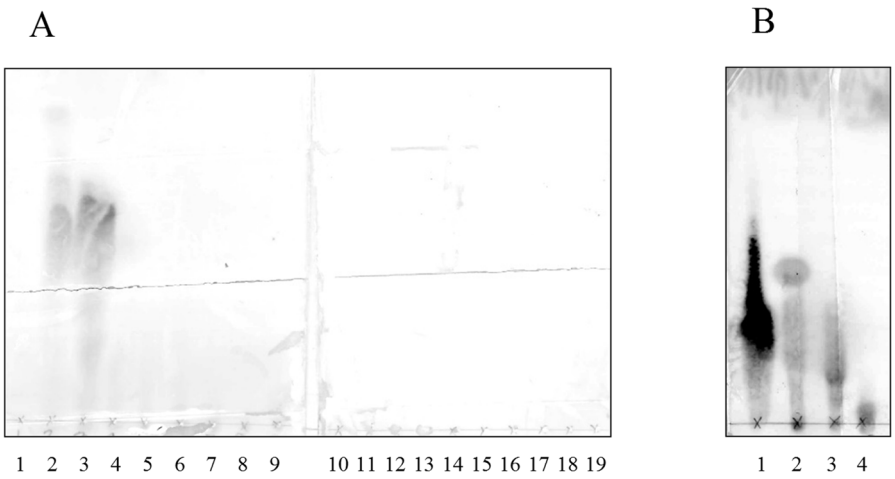

Fig. 4.12 Thin layer chromatography (TLC) of E1-1 fraction of *Q. phillyraeoides* ethyl acetate extract. Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of ethyl acetate:methanol:water (8:2:1).

A: According to the spot pattern, fractions 1 – 19 were combined as follows: E1-1-1 (Lanes 1 – 3), E1-1-2 (Lanes 4 – 5), E1-1-3 (Lanes 6 – 7), and E1-1-4 (Lanes 8 – 19).

B: Combined fractions were checked by TLC. E1-1-1 (Lane 1) fraction showed a dark spot, E1-1-2 (Lane 2) and E1-1-3 (Lane 3) fractions showed smeared spots, and E1-1-4 (Lane 4) showed no spot.

pattern, four fractions were combined as follows: E1-1-1 (Fr.1 – Fr.3), E1-1-2 (Fr.4 – Fr.5), E1-1-3 (Fr.6 – Fr.7), and E1-1-4 (Fr.8 – Fr.19) (Fig. 4.12A). These four fractions were checked by TLC (Fig. 4.12B). Among them, the E1-1-1 fraction showed a dark spot. E1-1-2 and E1-1-3 fractions showed smeared spots and E1-1-4 showed no spots. These fractions were examined for α-glucosidase inhibitory activities, and showed IC_{50} values of 6 μg/ml (E1-1-1), 21 μg/ml (E1-1-2), and 30 μg/ml (E1-1-3) (Table 4.8). E1-1-4 fraction showed no inhibitory effect on α-glucosidase (Table 4.8). E1-1-1 fraction showed the strongest inhibitory effect on yeast α-glucosidase with an IC_{50} value of 6 μg/ml. The E1-1-1 fraction was further purified by HPLC to provide the E1-1-1-1 fraction (QPE-1), which was collected at a retention time of around 8 (I) minutes as determined by UV

Table 4.8 Inhibitory effects of E1-1-1 – E1-1-4 fractions from E1-1 fraction of *Quercus phillyraeoides* ethyl acetate extract against yeast α-glucosidase.

Fraction	IC_{50} μg/ml
E1-1-1	6
E1-1-2	21
E1-1-3	30
E1-1-4	NA

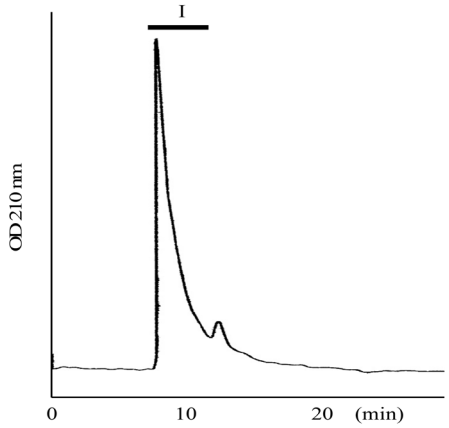

Fig. 4.13 High-performance liquid chromatography (HPLC) of E1-1-1 fraction of *Q. phillyraeoides* ethyl acetate extract. A peak was found at a retention time of around 8 (I) minutes by UV monitoring at 210 nm.

Fig. 4.14 Thin layer chromatography (TLC) of E1-1-1-1fraction of *Q. phillyraeoides* ethyl acetate extract. Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of hexane:ethyl acetate:methanol (8:2:1). Purified fraction E1-1-1-1 (QPE-1) showed a clear spot.

monitoring at 210 nm (Fig. 4.13). The purified fraction was also checked by TLC and showed a clear spot (Fig. 4.14). The E1-1-1-1 (QPE-1) fraction showed a strong inhibitory effect with an IC_{50} value of 1.6 µg/ml (Table 4.9).

The E2 fraction was divided into two fractions (E2-1 and E2-2) by HPLC, and an individual peak was found in fraction E2-1 collected at a retention time of around 8 (I)

Table 4.9 Inhibitory effects of E1-1-1-1 fraction from E1-1-1 fraction of *Quercus phillyraeoides* ethyl acetate extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
E1-1-1-1 (QPE-1)	1.6

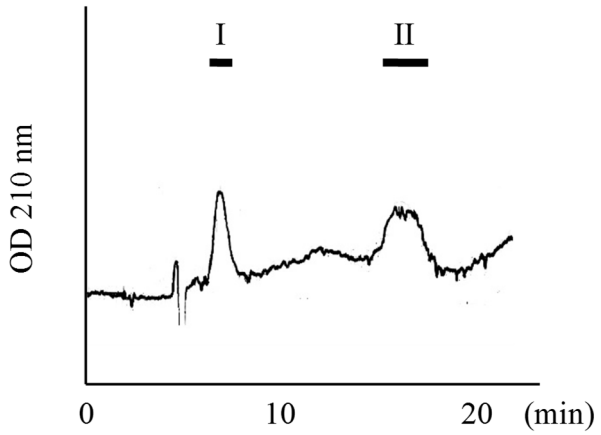

Fig. 4.15 High-performance liquid chromatography (HPLC) of E2 fraction of *Q. phillyraeoides* ethyl acetate extract. Peaks were found at retention times of about 8 (I, E2-1) and 16 (II, E2-2) minutes by UV monitoring at 210 nm.

Fig. 4.16 Thin layer chromatography (TLC) of E2-1fraction of *Q. phillyraeoides* ethyl acetate extract. Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of hexane:ethyl acetate:methanol (5:5:2). Purified fraction E2-1 (QPE-2) showed a clear spot.

minute as determined by UV monitoring at 210 nm (Fig. 4.15). The purified fraction was also checked by TLC and showed a clear spot (Fig. 4.16). The E2-1 (QPE-2) fraction showed significant inhibitory activity with an IC_{50} value of 26 µg/ml (Table 4.10).

Table 4.10 Inhibitory effects of E2-1 and E2-2 fractions from E2 fraction of *Quercus phillyraeoides* ethyl acetate extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
E2-1 (QPE-2)	26
E2-2	NA

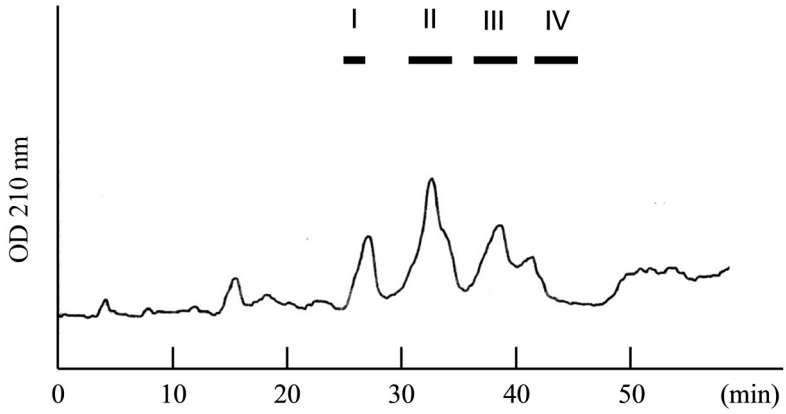

Fig. 4.17 High-performance liquid chromatography (HPLC) of E4 fraction of Q. phillyraeoides ethyl acetate extract. Four peaks were found in fraction E4 by UV monitoring at 210 nm

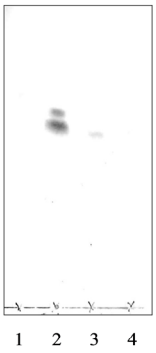

Fig. 4.18 Thin layer chromatography (TLC) of E4 fraction of *Q. phillyraeoides* ethyl acetate extract. Chromatographic fractions were checked by normal-phase thin layer chromatography (silica gel 60 F_{254}) using a mixture of hexane:ethyl acetate:methanol (5:5:2). E4-2 (Lane 2) fraction showed a clear spot, E4-1 (Lane-1) and E4-3 (Lane 3) fractions showed weak spots, and E4-4 showed no spots.

The E4 fraction was fragmented by HPLC and showed four peaks (E4-1 – E4-4) (Fig. 4.17). These four fractions were checked by TLC (Fig. 4.18). The E4-2 fraction showed a clear spot, E4-1 and E4-3 fractions showed faint spots, and E4-4 fraction showed no spots (Fig. 4.18). These fractions were examined for α-glucosidase inhibitory activities, and showed IC_{50} values of 55 μg/ml (E4 1) and 73 μg/ml (E4-2). E4-3 and E4-4 fractions showed no inhibitory effect on α-glucosidase activity (Table 4.11). The E4-2 fraction was purified by HPLC and fragmented into three fractions (E4-2-1 – E4-2-3),

which were collected at retention times of around 18 (I), 29 (II), and 31 (III) minutes as determined by UV monitoring at 210 nm (Fig. 4.19). Among them, the E4-2-1 fraction showed a clear peak. These fractions were examined for α-glucosidase inhibitory activities, and showed IC_{50} values of 27 µg/ml (E4-2-1), 33 µg/ml (E4-2-2), and 37 µg/ml (E4-2-3) (Table 4.12). The E4-2-1 (QPE-3) fraction showed strong inhibitory activity with an IC_{50} value of 27 µg/ml (Table 4.12).

Table 4.11 Inhibitory effects of E4-1 – E4-4 fractions from E4 fraction of *Quercus phillyraeoides* ethyl acetate extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
E4-1	55
E4-2	73
E4-3	NA
E4-4	NA

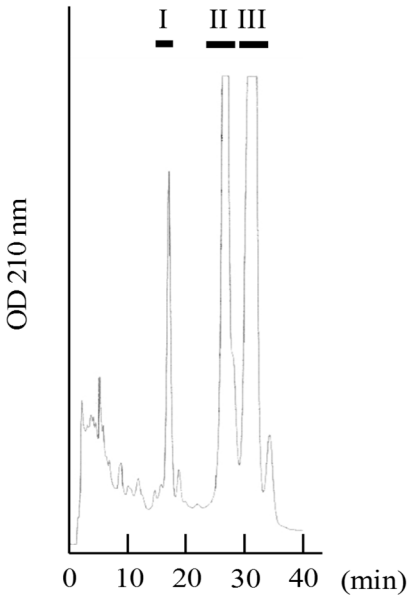

Fig. 4.19 High-performance liquid chromatography (HPLC) of E4-2 fraction of *Q. phillyraeoides* ethyl acetate extract. Three peaks were found in fraction E4-2 by UV monitoring at 210 nm. E4-2-1(I) fraction displayed a clear peak.

Table 4.12 Inhibitory effects of E4-2-1 – E4-2-3 fractions from E4-2 fraction of *Quercus phillyraeoides* ethyl acetate extract against yeast α-glucosidase.

Fraction	IC_{50} µg/ml
E4-2-1 (QPE-3)	27
E4-2-2	33
E4-2-3	37

4.4 Discussion

4.4.1 *T. chebula*

The butanol extract of *T. chebula* showed high yeast α-glucosidase inhibitory activity in the screening experiments. The butanol extract was fractionated into seven fractions (B1 – B7) by silica gel column chromatography. Among them, the B1 fraction showed the strongest inhibitory activity. On HPLC, the B1 fraction showed two active peaks, designated as B1-1 (TCB-1) and B1-2 (TCB-2) (Fig. 4.1). B1-1 (TCB-1) and B1-2 (TCB-2) fractions showed significant inhibitory effects against α-glucosidase, with IC_{50} values of 2 μg/ml and 3 μg/ml, respectively (Table 4.2).

T. chebula contains various phytoconstituents, such as alkaloids, flavonoids, glycosides, phenolic compounds, saponins, steroids, tannins, and quinine (Baliah and Astalakshmi, 2014). These active constituents have been applied as α-glucosidase inhibitors in many previous studies. Alagesan *et al*. (2012) reported that phenolic compounds of plants inhibited α-glucosidase enzyme activity. The results of the present study suggested that isolated fractions of *T. chebula* may contain compounds with α-glucosidase inhibitory activities.

4.4.2 *M. japonicus*

The butanol extract of *M. japonicus* showed high yeast α-glucosidase inhibitory activity in the screening experiments. The butanol extract was fractionated into seven fractions (B1 – B7) by silica gel column chromatography, among which the B2 fraction showed the highest inhibitory activity. On MPLC, the B2 fraction was divided into five fractions (B2-1 – B2-5), and HPLC of the B2-4 fraction showed an active peak with an IC_{50} value of 10 μg/ml (B2-4-2; MJB 1). Li *et al*. (2009) reported that the flavonoids extracted from *Crataegus monogyna* (Hawthorn) leaves had an IC_{50} value of 7.1 μg/ml, and suggested that the extract contained the bioactive compounds, quercetin (flavonol) and vitexin (flavone) with α-glucosidase inhibitory activities. In the present study, the MJB-1 fraction isolated from *M. japonicus* showed an IC_{50} value of 10 μg/ml, which was close to that of the *C. monogyna* (Hawthorn) leaf flavonoid extract. This fraction may contain bioactive antihyperglycemic compounds.

4.4.3 *Q. phillyraeoides*

The ethyl acetate extract of *Q. phillyraeoides* displayed the highest α-glucosidase inhibitory activity in the screening experiments. The ethyl acetate fraction of *Q. phillyraeoides* was divided into six fractions by MPLC, among which the fractions E1 – E4 shoed excellent inhibitory effects. Before purification, the E2 fraction showed higher inhibitory activity (IC_{50} value: 14 μg/ml, Table 4.6) than the E2-1 fraction (IC_{50} value: 26 μg/ml, Table 4.10). These different inhibitory effects may have been because multiple components of the E2 fraction worked together, whereas the E2-1 fraction had a single component that worked alone. Repeated column chromatography of the ethyl acetate extract of *Q. phillyraeoides* (E1) fraction yielded E1-1-1-1 (QPE-1), which exhibited strong inhibitory activity (IC_{50}: 1.6 μg/ml). On the other hand, Dewi *et al*. (2007) reported that the ethyl acetate extract of koji *Aspergillus terreus* fractionated by column chromatography and purified fraction F10-4 exhibited high inhibitory activity with an IC_{50} value of 2.9 μg/ml. These findings suggested that the α-glucosidase inhibitory activity of *Q. phillyraeoides* (E1-1-1-1) was stronger than that of koji *Aspergillus terreus* (Fr.10-4), which has been identified as a strong α-glucosidase inhibitor.

Six fractions (TCB-1, TCB-2, MJB-1, QPE-1, QPE-2, and QPE-3) isolated from *T. chebula* (butanol), *M. japonicus* (butanol), and *Q. phillyraeoides* (ethyl acetate) leaf extracts were assayed for α-glucosidase inhibitory effects *in vitro*. Six isolated fractions exhibited strong inhibitory effects against α-glucosidase (Table 4.13). Wu *et al*. (2012) reported that six isoflavones, i.e., swertisin (119 μg/ml), 2"-*O*-rhamnosylswertisin (333 μg/ml), genistein (74 μg/ml), genistin (83 μg/ml), mangiferin (112 μg/ml), and daidzin (97 μg/ml), showed strong inhibitory effects against α-glucosidase activity, consistent with the results of the present study.

Table 4.13 Effects of methanol extracts of the leaves of nineteen plants against yeast α-glucosidase

Plant name	Purified fractions	IC_{50} (μg/ml)
Terminalia chebula	TCB-1	2
	TCB-2	3
Mallotus japonicus	MJB-1	10
Quercus phillyraeoides	QPE-1	1.6
	QPE-2	26
	QPE-3	27
Acarbose (Positive control)		13,000

We evaluated the inhibitory activities of six fractions (TCB-1, TCB-2, MJB-1, QPE-1, QPE-2, and QPE-3) against yeast α-glucosidase in comparison with the known α-glucosidase inhibitor, acarbose. All of the isolated fractions exhibited higher inhibitory activity than acarbose. Li *et al*. (2009) reported similar results where six compounds (apigenin, vitexin, isovitexin, luteolin, orientin, and isoorientin) showed stronger inhibitory effects than acarbose, which was compatible with the findings of the present study.

4.5 Conclusions

Six fractions (TCB-1, TCB-2, MJB-1, QPE-1, QPE-2, and QPE-3) were isolated from *T. chebula* (butanol), *M. japonicus* (butanol), and *Q. phillyraeoides* (ethyl acetate) leaf extracts. Among these fractions, QPE-1, TCB-1, and TCB-2 exhibited excellent α-glucosidase inhibitory activities. The results of these experiments indicated that *Q. phillyraeoides* and *T. chebula* are candidates as sources of potential antidiabetic compounds. Further *in vivo* studies are necessary to confirm these observations.

Chapter 5
General Discussion

In Bangladesh, many medicinal plants are used to treat various types of disease. About 80% of the rural people are dependent on traditional medicine for their primary healthcare. However, very few plants are used to control diabetes. The rate of diabetes is increasing among people over 50 years old in Bangladesh, but the number of diabetes patients in Bangladesh is still not high. However, the number of patients with diabetes will likely increase in future because of the rapid growth of the Bangladeshi economy. Therefore, the new medicinal plants are required for the treatment of diabetes in Bangladesh. We screened the leaf extracts of three Bangladeshi medicinal plant species, i.e., *E. officinalis*, *T. bellirica*, and *T. chebula*, for antidiabetic effects. The results presented here confirmed that the leaf extracts of these three Bangladeshi medicinal plants showed significant inhibitory effects against carbohydrate digestive enzymes.

Traditional medicine is occasionally used in Japan. In this study, 19 types of Japanese woody plant leaves were screened for carbohydrate digestive enzyme inhibitory effects, and to identify the structures of active constituents to produce modern medicines. Five plants, i.e., *E. sylvestris var. ellipticus*, *M. japonicus*, *M. rubra*, *Q. phillyraeoides*, and *S. sebiferum*, showed strong inhibitory effects against yeast α-glucosidase activity.

Finally, the butanol extract of *T. chebula*, butanol extract of *M. japonicus*, and ethyl acetate of *Q. phillyraeoides* were examined to purify the carbohydrate digestive inhibitors. Six fractions (TCB-1, TCB-2, MJB-1, QPE-1, QPE-2, and QPE-3) were isolated from *T. chebula* (butanol extract), *M. japonicus* (butanol extract), and *Q. phillyraeoides* (ethyl acetate extract) leaf extracts. Among these fractions, QPE-1(*Q. phillyraeoides*), TCB-1 (*T. chebula*), and TCB-2 (*T. chebula*) exhibited excellent α-glucosidase inhibitory activities. Based on the results of these studies, *Q. phillyraeoides* and *T. chebula* are candidates as possible sources of antidiabetic compounds. Further *in vivo* studies are necessary to confirm these observations.

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List of main papers

Main papers used in creating the dissertation

Peer-reviewed papers

1. Author name (published year), Title, Journal name, volume: page numbers.

2 Author name (published year), Title, Journal name, volume: page numbers.

Additional papers

1. Author name (published year), Title, Journal name, volume: page numbers.

2 Author name (published year), Title, Journal name, volume: page numbers.

Conference presentations

1. Speakers, Title, Conference name and location, Date of presentation

2. Speakers, Title, Conference name and location, Date of presentation