Chapter 1
Introduction

1.1 Introduction

The worldwide problem of type-2 diabetes mellitus and ever-increasing problem of obesity in the developed and developing countries are correlated with the ingestion of high-calorie foodstuffs and sedentary lifestyles [10]. Foods with higher resistant starch (RS) proportion and low glycemic index (GI) are beneficial for preventing obesity, insulin resistance, and diabetes owing to their potential to maintain blood sugar levels and its higher satiety value [8]. Polyphenols have shown to hinder starch digestive enzymes and are regarded as a potential regulator for glucose uptake and metabolism [3]. Amongst the various promising options to reduce the digestibility of carbohydrates is by mixing starch and polyphenols, where they can interact covalently or non-covalently, thereby altering the glycemic responses of carbohydrates. Over the last few years, an increasing attention in the application of polyphenols to develop starch-polyphenol complexes, which is a promising option to challenge the current issues of obesity and diabetes has been observed [45]. Furthermore, the starch-polyphenols complex exhibited the dual property of resistant starch along with the bioactivities of the attached polyphenol. Starch-polyphenols complexes are less susceptible to starch digesting enzymes *i.e.*, α-amylase and α-glucosidase enzymes [10, 11].

Polyphenols liberated from cellular compartments during processing were interacted with starch during food processing leading to the development of starch-polyphenol complex and alter the structural and physicochemical properties of starch [8, 23]. Thus, the polyphenols release from the *Euryale ferox* seed shell during processing due to tissue rupture, mixing, and heating, are expected to interact with the kernel starch, ultimately affecting the characteristics of starch. Although the ultimate goal of starch modification with polyphenols is to lower starch digestibility and enhance the bio accessibility of phenolics, the complexation unavoidably caused alterations to the characteristics of starch. The characteristics of these complexes is determined by the type of starch, complexation method, type, and concentration of polyphenols [45]. Therefore, the main intention of the present investigation was carried out to ascertain the impact of polyphenols on the digestibility of *Euryale ferox* kernel starch.

1.2 *Euryale ferox*

1.2.1 Distribution of *Euryale ferox*

Euryale ferox (Fig. 1.1), an aquatic annual crop, is the single species in the genus *Euryale* of the Nymphaeaceae family [28]. It is commonly known as fox nut, gorgon nut; prickly water lily; black diamond etc. [28]. In India, *Euryale ferox* is commonly known as makhana. The word makhana is made up of two Sanskrit words- "Makh" which means sacrifice, and "Anna" meaning grain. Gorgon nut is considered auspicious and consumed during religious ceremonies in India [20]. *Euryale ferox* is known as nikori in Assamese; thangjing in Manipuri. *Euryale ferox* is native of South-East Asia and is distributed in Bangladesh, Nepal, Korea, Japan, China, North America, Russia, and India. It is believed that *Euryale ferox* was once distributed in India, covering a long-range from Kashmir to Manipur alongside the Himalayan stretch [20]. It has adapted to the tropical climate of India and presently grown in eastern and north-eastern part of India including Manipur, West Bengal, Bihar, Assam, Tripura, Jammu and Kashmir, Uttar Pradesh, Tripura, Madhya Pradesh, Odisha and Rajasthan. In addition to being an important crop for aquatic ecosystems, *Euryale ferox* is generally cultivated for its starchy edible seeds in India, China, and south East Asian countries. In India, commercial cultivation of *Euryale ferox* is confined to Manipur, Bihar, West-Bengal, and Madhya Pradesh [25].

Fig. 1.1 (a) *Euryale ferox* fruit; **(b)** Cross section of *Euryale ferox* fruit

1.2.2 Description of *Euryale ferox*

Euryale ferox plant is characterized by its bright green leaves of 30 to 120 cm diameter with a short thick rhizome which float over water surface. The broad, thorny, elliptic or orbicular mature leaves are quilted in texture on the upper side and sharp spines all over the surfaces. The ventral side of the leaves are green in color while the ventral sides are red or purple. The veins are highly swollen on the dorsal side and bear many sharp pines on the leaf stalk. The flower is complete, solitary, big, up to 4-5 cm long with long pedicel having four sepals covered with large spines, petals are bright purple in color paling to white in the center. The color of the sepal is royal purple on the reverse [20].

1.2.3 *Euryale ferox* seed

Euryale ferox is a spongy fruit crowned with everlasting sepals that measure around 5-8 cm in diameter, and covered by spines all over the surface. Each fruit contains 20-200 pea-sized seeds of hard black seed coats with a diameter ranging from 0.5 cm to 1.5 cm. The seed is composed of three portions: the inner kernel with brown or black bran layer, the outer shell and covered by bright streaked membranous aril. The major edible part of the nut is its starchy kernel, which makes up roughly 70% of the weight of the kernel [25]. The seeds are loaded with numerous macro and micro nutrients such as essential amino acids, essential oil, carbohydrates, vitamin B, vitamin C, minerals *viz.*, phosphorus, potassium, calcium, iron, zinc, and other trace elements [20].

Euryale ferox seed shell, which accounts for half of the seed, contains a substantial amount of polyphenols, but are usually discarded [28]. Total phenolic content in seed shell extracts was reported to be around 114 mg/g in dry weight basis [44]. *Euryale ferox* seed shells contain less water, thus it can be easily dehydrated and further exploited as a source of phenolic compounds [49]. Major bioactive components present in *Euryale ferox* are pyrogallol, gallic acid, chlorogenic acid, epicatechin, rutin, flavonoids, sterols, tocopherol trimers, cerebrosides, cyclic dipeptides, sesquineolignans, sesquiterpene, tannins etc. [44,49]. Epidemiological studies have shown that *Euryale ferox* seed shell extract could alleviate chronic ailments including type 2 diabetes, heart disease, and nephropathy because of its antioxidant, anti-hyperlipidemic, and anti-inflammatory properties [1, 24 25, 35, 44].

1.3 Starch

Starch is a key storage carbohydrate found in cereals, pulses, roots, tubers, and unripe fruits [29]. It is the primary constituent of human diet, where it accounts for the major share of energy [30]. The linear amylose and highly branched amylopectin molecules make up this natural polymer. The linear chains of these two polymers are formed by α-(1,4)-glycosidic bond, while the branches of α-D-glucose are connected by both α-(1,4) and α-(1,6) glycosidic linkages [21]. Starch is a versatile and inexpensive biopolymer that is used in a variety of sectors, including food, pharmaceuticals, paper, and textiles industries. Starch has a wide range of applications in the food processing industry, including bulking, thickening, stabilizing, gelling, encapsulating, texture improvement, and humectants [4].

Tubers (cassava, potato, and sweet potato) and cereal starches (maize, wheat, and rice) have dominated research and industrial applications to date. In recent years, non-conventional and underutilized starch sources with desired physicochemical and functional features that do not require genetic or chemical modification are being investigated for their possible usage in order to meet the growing demand for starch [36]. The physicochemical and functional properties of starches separated from different raw materials are influenced by their botanical origin, granule shape, crystallinity, and amylose content [42]. Therefore, before approving a novel source of starch for use in industries, a significant amount of effort must be done in order to understand its structural, physicochemical, and functional qualities so as to suggest most appropriate applications in food and non-food industries [41].

1.4 Resistant Starch

Starch, the principal energy source in human nutrition, is abundant in almost all staple foods [45]. Although raw starches contain an appreciable amount of resistant starch, they are boiled before consumption, making them readily digestible, causing glycemic fluctuation, which is undesirable, compelling to design novel starch with low digestibility for a slow and steady release of glucose. With a growing interest in health and nutrition, consumers are looking for food that has the potential to protect against lifestyle-related diseases. As a result, food manufacturers and researchers have focused on improving the nutritional properties of starch, which is the primary ingredient of convenience foods [16].

Nutritionally, starch is categorized into three types: The portion of starch that can be quickly broken down and assimilated in the small intestine is known as rapidly digestible starch (RDS), and it promptly raises blood sugar levels. Owing to its steady release of glucose in the lower small intestine, the SDS (slowly digestible starch) is considered beneficial for maintaining post-prandial blood glucose level. Whereas, resistant starch (RS), is a portion of starch that escapes enzymatic hydrolysis in the small intestine instead metabolized by gut microbiota, thereby producing short-chain fatty acids *viz*., propionate, butyric acid, acetic acid, thus helps in maintaining gut health [38]. Therefore, increasing intake of resistant starch in daily diets is considered to be a promising approach to reduce relative potential health risks associated with high glycemic index, as it will help to regulate blood glucose level and corresponding insulin release. One of the promising ways to reduce the digestibility of starch is by adding natural plant extracts rich in polyphenols, as they can interact covalently or non-covalently with starch, thus modulating the glycemic responses of carbohydrates [8]. Moreover, incorporating phenolic-rich fractions like cereal bran and phenolic extracts have increased the bio accessibility and bioavailability of phenolics in food matrices [3].

1.5 Polyphenols

Polyphenols are secondary metabolites produced by plants. Polyphenols exhibited numerous biological activities, such as antioxidant, anti-inflammatory, antibacterial, and antiviral activities. Polyphenols can help prevent chronic diseases like cancer, diabetes mellitus, osteoporosis, and neurodegenerative ailments by curtailing the level of oxidative impairment to carbohydrates, proteins, lipids, and DNA in living cells and tissues. They also aid in the avoiding chronic disorders like cancer, diabetes, osteoporosis, and neurodegenerative ailments. Furthermore, polyphenols have been shown to inhibit starch digesting enzymes and are regarded as a potential regulator for glucose uptake and metabolism. However, polyphenols are sensitive to pH, temperature, light and unpleasant taste which limits their applications [3]. Polyphenols interacted with α-amylase and/α-glucosidase, mainly through non-covalent bonding, such as hydrophobic or hydrogen bonding (between the hydroxyl portion of polyphenols and the active binding position of enzymes). The binding of polyphenols to active sites of enzymes caused conformational changes in the enzyme, suppressing their activity and preventing starch from reaching

them. These interactions will help to stabilize the binding between the enzyme and the polyphenols, creating a complex that leads to decrease α-amylase function [42].

The capability of polyphenols to alter the physicochemical properties of starch and act as physical barriers between enzymes and starch. The complex formation by non-covalent bonding, especially hydrogen bonding, improves starch structural properties due to the formation of impermeable networks during gelatinization which covered on the surface of starch granules, restricting direct accessibility of enzyme. However, it is difficult to establish whether the polyphenols adsorbed onto starch have an inhibitory effect on enzymes or not [27, 30]. Therefore, the application of polyphenols to interact with starch and produce starch polyphenol complexes with reduced starch digestibility is a promising strategy to challenge the current issues [13, 45].

1.6 Starch-polyphenol complex

Polyphenols are extensively studied for its ability to inhibit starch digesting enzymes and are also regarded as a potential regulator for glucose uptake and metabolism [3]. Over the last few years, we have observed an increasing attention in the application of polyphenols to produce starch-polyphenol complexes because of its distinctive physicochemical and functional properties, which is a promising strategy to challenge the current issues of obesity and diabetes [45]. The effects of tea polyphenols [26], flavonoids from lotus leaf [42] on the attributes of starch have been reported.

1.7 Pre-gelatinization method

Pre-gelatinization is the most commonly employed physical technique for producing starch– polyphenol complex amongst all the existing techniques [45]. This method facilitates a rapid and safe non-covalent attachment of polyphenols to starch without the use of organic solvents or hazardous radical initiators [27]. During this process, starch is mixed with water to create a form a suspension. The suspension is then briefly cooked at a specified temperature and then polyphenols were added. The suspensions of the mixtures were typically heated for 20 min to 4 h. When starch is gelatinized, it releases amylose, making it a proper environment for complexation [16]. If polyphenols are introduced in this condition, the phenolics tend to dissolve and interact with the starch to develop complexes. Amylopectin is less likely to form complexes with polyphenols because the highly branched structure of amylopectin behaves as a steric hindrance. In most of cases,

non-covalent bonding interactions, especially hydrophobic bonding, hydrogen bonding, and van der Waals forces, are frequently accountable for attaching the hydroxyl group of polyphenols with starch molecules [10,11].

1.8 Diabetes mellitus

Diabetes mellitus is a long-term metabolic abnormality distinguished by abnormal blood sugar level due to insulin resistance or insufficient production. The outburst of type 2 diabetes mellitus (T2-DM) is increasing globally, and this phenomenon is alarming. The primary goal of anti-diabetic drug is to control prolonged hyperglycemia, which otherwise is linked to micro- and macrovascular rupture thereby causing cardiopathy, neuropathy, nephropathy, and retinopathy [5, 32]. New pharmacological targets for the management of T2-DM includes inhibiting α-glucosidase or α-amylase, DPPIV enzymes. DPPIV inhibitors has the potential to lower post-meal glucose levels, confirming that DPPIV inhibitors are effective, and well-tolerated therapies for T2-DM [34].

1.9 DPP IV inhibition

Dipeptidyl peptidase IV, also referred to as "gliptins," is a soluble plasma enzyme of the intestinal epithelium, kidney, and liver [5] and lung, which inactivates ‘GLP-1’ commonly known as glucose-dependent insulinotropic polypeptide and ‘GIP’ or glucagon-like peptide-1 into inactive form. This enzyme is a member of the serine proteases family, having 766 amino acids and Asp-His-Ser at the active site. The GIP and GLP-I, are generated by the intestinal mucosa's L and K cells respectively are the significant incretin hormones responsible for maintaining blood sugar levels [Bharti et al., 2012]. Under normal metabolic condition, GLP-I stimulates the function of α- and β-cells, insulin production, and glycogenesis in the muscles and liver, slowing down gastric emptying and thereby regulating postprandial glucose spikes [6, 33].

DPP IV cleaves the alanine and proline of GLP-1 and GIP in their N-terminal ends. However, in T2DM, incretin activity is weakened, GLP-I and GIP become ineffective. Increased GIP and GLP-1 synthesis as well as the inhibition of DPP-4 are crucial mechanisms that are clinically relevant for controlling hyperglycemia in type 2 diabetes. Therefore, administration of DPP IV inhibitors suppresses the enzyme, thus stimulating GLP-1 and GIP [33], inhibits glucagon release and enhances the activity of β-cells [5]. Several plant extracts have been an age-old treatment option for diabetes, and there is

surging interest in exploring them as a source of pharmaceuticals. Moreover, the use of DPP IV inhibitors from herbs are increasing since they that have less side effects, less expensive, and are easily available [5].

1.10 Inhibition of α-amylase and α-glucosidase

The enzyme α-amylase breaks starch into α-dextrins, maltotriose and maltose, which are again digested by α-glucosidase and releases sugar, which is sent into the bloodstream and causes plasma glucose to spike. Therefore, reducing the activities of α-amylase and α-glucosidase, will delay the postprandial spike of serum glucose and is an efficient strategy to regulate postprandial hyperglycemia [46]. The inhibition potential of polyphenols against α-glucosidase varied because of the differences in their molecular structures and binding sites with the enzymes. The binding of polyphenols to active sites of enzymes caused conformational changes in the enzyme, suppressing their activity and preventing starch from reaching them.

Polyphenols interacted with α-amylase and/α-glucosidase, mainly through non-covalent bonding, such as hydrophobic or hydrogen bonding. These interactions will help to stabilize the binding between the enzyme and the polyphenols, creating a complex that leads to decrease α-amylase activity [30]. The α-amylase suppression actions of plant extracts are stronger when the solvent has a lower polarity [39]. Polarity of the solvent has an impact on the concentration of active components in the extract. Selection of most appropriate solvent is a critical step for phytochemicals extraction [40]. Because different active compounds dissolve differently in different solvents. The chosen solvent must be able to fully extract the desired compounds and must not induce any chemical changes to them [33].

1.11 Glucose uptake assay

The primary insulin-responsive glucose transporter is GLUT-4. In type 2 diabetes mellitus, the activity of Glut-4 proteins is downregulated due to insulin resistance, which in turn led to reduced glucose uptake. Therefore, GLUT-4 is regarded as one of the most therapeutically promising targets for the management of T2-DM due to its significance in maintaining glucose homeostasis [18]. The skeletal muscle is one of the crucial tissues associated with the regulation of the post-prandial glucose level by improving the transfer of glucose from the blood into the muscle cell, mediated by glucose transporter type 4

(GLUT-4) [18]. L6 and 3T3-L1 cell lines are the most appropriate biological models for studying glucose absorption and GLUT4 translocation. Insulin boosts glucose absorption in skeletal tissue, by increasing the number of functional plasma membrane. Skeletal muscle and adipose tissue are now widely recognized as key target sites for glucose digestion, as well as maintenance of glucose homeostasis, and key targets for insulin-induced glucose uptake to regulate hyperglycemia [48].

1.12 Anti-inflammatory activity

Inflammation is primarily a physiological defense, an immunological and cytoprotective immune response to oxidative stress due to allergens, toxins, or microbes [2]. Uncontrolled acute inflammation, however, lead to chronic inflammation, resulting several chronic inflammatory diseases, including cancer, autoimmune diseases, hypertension, and atherosclerosis [37]. Inflammatory illnesses have been treated using plants or products made from plants [31]. Most plant-derived compounds including phenolic, flavonoid, and terpenoids reduces oxidative stress, and suppress the carbohydrate digestive enzymes [22].

Pro-inflammatory mediators like tumor necrosis factor (TNF)-α, IL-18, IL-12, IL-6, are upregulated in both acute and chronic inflammatory diseases while COX-2 promote the secretion of pro-inflammatory intermediary. In the current study, THP-1 cells have been used to examine the immunomodulatory effects of EFSSE. THP-1 cells, which are obtained from the monocytes of human leukemia, have been widely employed to examine the immune response mechanisms of monocytes and monocyte-derived macrophages in the immune system [2]. Lipopolysaccharides (LPS), which make up a large portion of the cell wall of Gram-negative bacteria are triggered directly or indirectly by host-derived mediators including chemokines, cytokines, and serine proteases etc. When LPS are stimulated, the regulatory proteins in THP-1 cells trigger inflammation to initiate [37].

1.13 Bread

Bread is regarded as one of the best carriers for food fortification, supplementation, and enrichment due to its economical, availability, popularity, and extensive consumption. Moreover, bread is a carbohydrate-rich, high glycemic index (GI) staple food consumed all over the world; hence reformulating bread to lower its glycemic load is of great interest [14].

1.14 Gluten free bread

Gluten is recognized as the primary ingredient in the baking as it responsible for the dough elasticity, resistance to stretching, tolerance during mixing, and gas retention [19]. Because of the lack of gluten, the adhesiveness between the ingredients is reduced and cannot form a viscoelastic system, resulting in poor texture quality. As compared to the conventional bread flour, gluten free flour generally had poorer nutritional quality in terms of low dietary fiber, resistant starch (RS) content, vitamins, minerals, bioactive compounds but higher glycemic index (GI) [9]. In the recent years, there has been a remarkable research attempt to improve the physical, structural, as well as nutritional properties of gluten-free breads by incorporating a wide range of ingredients, such as pregelatinized starch [19], dietary fiber, resistant starch, and modified starch [50].

1.15 Swarm intelligence supervised neural network (SISNN)

The impact of polyphenols on glycemic control may be predicted through *in-vitro* study. Starch digestion kinetics may be studied based on the first-order reaction kinetics, and then predicted glycemic index (pGI) can be determined [15]. For more adequate and accurate predictions, neural network approaches can be applied. A neural network helps to model input and output variables based on the training, testing, and validation approaches. The neural network executes its prediction mapping in association with weight bias values and activation function. Swarm intelligence, or in generic terms particle swarm optimization (PSO), is an evolutionary approach for the optimization of process parameters. A neural network optimized based on PSO helps to develop a stronger prediction capacity for applying the black-box modeling approach [7]. A hybrid technique based on swarm intelligence and artificial neural network, known as swarm intelligence supervised neural network (SISNN), along with mathematical modeling approaches may be employed for better starch digestion kinetics. Principal component analysis (PCA) is an effective tool to decrease the number of dependent variables (i.e., characteristics) to a lesser number of underlying variables (called factors) based on the patterns of connection amongst original variables data, trying to extract the most important elements amongst the variables while maintaining the most original variable information [12].

1.16 Hypotheses

I. We hypothesized that during the hydrothermal processing of *Euryale ferox* seed, polyphenols may transfer from the shell into the starchy kernel and modulate its digestibility.
II. The activity of the *Euryale ferox* seed shell extract for the management of diabetes may be mediated by the compounds present in the shell extract which inhibits carbohydrate digestive enzymes along with its antioxidant and anti-inflammatory activity.
III. The starch-polyphenol complex may have the synergistic impact of both polyphenol and resistant starch.

1.17 Gap of Research

- There has been relatively little work on the isolation and characterization of *Euryale ferox* kernel starch.
- The scientific mechanism for the antidiabetic, anti-inflammatory activity of *Euryale ferox* seed shell extract has not been reported.
- The effect of *Euryale ferox* seed shell extract addition on the physicochemical, nutritional and organoleptic properties of the end product has not been reported.
- The effect of starch-polyphenols complex incorporation on the physicochemical, nutritional and organoleptic properties of the food product has not been reported.

1.18 Objectives

I. To extract and characterize starch of the kernel of *Euryale ferox* seed
II. To extract polyphenols from the shell of *Euryale ferox* seed and evaluate its bioactivities
III. To develop and characterize starch-polyphenol complex from *Euryale ferox* seed
IV. To incorporate starch-polyphenol complex from *Euryale ferox* seed in bread formulation

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