In Vitro Assessment of the Antidiabetic Potential of Medicinal Rice Varieties Through Biochemical Assays and Gene Expression Analysis

Dissertation submitted to the University of Kerala in partial fulfilment of the requirements for the award of the Degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

Submitted by

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T.K.M. COLLEGE OF ARTS AND SCIENCE, KARICODE, KOLLAM
JULY, 2026

Department of Biochemistry

T.K.M. College of Arts and Science

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CERTIFICATE

This is to certify that the dissertation entitled **'In Vitro Assessment of the Antidiabetic Potential of Medicinal Rice Varieties Through Biochemical Assays and Gene Expression Analysis'** submitted to the University of Kerala in partial fulfilment of the requirements for the award of the degree of Master of Science in Biochemistry, is a record of original research work carried out by **FATHIMA N. (Candidate code: 63824142004)** of the Department of Biochemistry, T.K.M. College of Arts and Science, Kollam under the guidance and supervision of **Prof. (Dr.) R. Rajalakshmi & Dr. Nadiya F.,** in the Inter University Centre for Genomics and Gene Technology (IU-CGGT), University of Kerala, Kariavattom. It is also certified that no part of this work has been presented for the award of any other degree, diploma, or similar title at any other university.

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Declaration

I hereby declare that the thesis titled "**In vitro Assessment of the Antidiabetic Potential of Medicinal Rice Varieties Through Biochemical Assays and Gene Expression Analysis"** is based on original research work carried out by me under the supervision of **Prof. (Dr.) R. Rajalakshmi** and **Dr. Nadiya F.,** in the Inter University Centre for Genomics and Gene Technology (IUCGGT), University of Kerala. I further declare that no part of this work has been presented for the award of any other degree, diploma, or similar title from any other university.

FATHIMA N.

Acknowledgements

The successful completion of this dissertation is due to the guidance, encouragement, and support of many individuals. I would like to express my heartfelt gratitude to the Almighty for blessing me with good health, strength, wisdom, and perseverance throughout my postgraduate studies and during the completion of this research work.

I sincerely thank **Prof. (Dr.) R. Rajalakshmi**, Hon. Director, Inter University Centre for Genomics and Gene Technology (IUCGGT), for granting me the opportunity and providing the facilities required to carry out this research at the centre.

I owe my deepest gratitude to my research guide, **Dr. Nadiya F.**, Research Associate, IUCGGT, whose constant guidance, expert suggestions, patience, and encouragement inspired me throughout this study. Her support and valuable insights have been invaluable in the successful completion of this dissertation.

I especially thank my beloved principal, **Prof. (Dr). M. J. Sheeba,** T.K.M. College of Arts and Science, Karicode, Kollam, for providing me with such a wonderful platform to complete my project.

I am extremely thankful to Smt. **Dr. Ajinza A.,** Head of the Department of Biochemistry and faculties **Dr. Ansil P. N., Dr. Soumya S., Dr. Sumayya A.S. and Dr. Hari Sankar H.S.**, for the critical guidance and encouragement.

It would be beyond words if I try to express my thanks to **Dr. Lakshmi N.,** Research Associate, **Ms. Arya C.S.**, and **Mr. Sajith S. K.,** JRFS, IUCGGT, **Ms. Mihina B.T.** who were most welcoming, cooperative and helpful throughout the course of my work.

I wish to pay my gratitude to my loving parents and friends for their constant encouragement and moral support given throughout my study period.

FATHIMA N.

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Abbreviations

2-DG6P	: 2-Deoxy-D-Glucose-6-Phosphate
BSA	: Bovine Serum Albumin
cDNA	: Complementary DNA
ddH_2O	: Double-Distilled Water
DEPC	: Diethyl Pyrocarbonate
DMEM	: Dulbecco's Modified Eagle Medium
DMF	: Dimethylformamide
DMSO	: Dimethyl Sulfoxide
DNS	: 3,5-Dinitrosalicylic Acid
DTNB	: 5,5′-Dithiobis-(2-nitrobenzoic Acid) (Ellman's Reagent)
FBS	: Fetal Bovine Serum
HEPES	: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
IC_{50}	: Half Maximal Inhibitory Concentration
IRS	: Insulin Receptor Substrate
KH_2PO_4	: Potassium Dihydrogen Phosphate
KRPH	: Krebs–Ringer Phosphate HEPES Buffer
L6	: Rat Skeletal Muscle Cell Line (L6 Myoblasts)
HepG2	: Human hepatocellular carcinoma cell line
$MgSO_4$	: Magnesium Sulfate
MTT	: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NAD(P)H	: Nicotinamide Adenine Dinucleotide (Phosphate), Reduced Form
NF-κB	: Nuclear Factor Kappa B
PBS	: Phosphate-Buffered Saline
T2DM	: Type 2 Diabetes Mellitus

Abstract

The increasing prevalence of diabetes has created a need for safe and effective natural alternatives that can help regulate blood glucose levels. Traditional medicinal rice varieties of Kerala, particularly Njavara and Rakthashali, have long been used in Ayurveda for their therapeutic benefits. However, scientific evidence supporting their antidiabetic properties is limited. Therefore, the present study was undertaken to evaluate the in vitro antidiabetic potential of methanolic extracts of Njavara and Rakthashali rice varieties.The antidiabetic activity of the rice extracts was evaluated using α-amylase inhibition, MTT cell viability, glucose uptake, and quantitative real-time PCR (qRT-PCR) analysis of the GLUT4 gene. The α-amylase inhibition assay was performed to assess the ability of the extracts to inhibit carbohydrate digestion, while the MTT assay in HepG2 and L6 cell lines was used to determine safe concentrations for further studies. The glucose uptake assay was carried out in L6 skeletal muscle cells, and the optimum concentration (100 µg/mL) identified from this assay was used to evaluate GLUT4 gene expression by qRT-PCR using GAPDH as the internal reference gene.

Both methanolic extracts exhibited significant concentration-dependent antidiabetic activity. Njavara showed higher α-amylase inhibition (76.4%) than Rakthashali (69.5%), indicating a greater ability to reduce carbohydrate digestion and postprandial glucose release. Both extracts maintained good cell viability at lower concentrations and significantly enhanced glucose uptake in L6 cells, with Njavara (1.95 µM) showing slightly higher uptake than Rakthashali (1.62 µM). Gene expression analysis further demonstrated that both extracts significantly increased GLUT4 expression, with Njavara producing approximately 1.90-fold and Rakthashali 1.56-fold higher expression than the untreated control. The enhanced GLUT4 expression was consistent with the increased glucose uptake observed in L6 cells, suggesting improved glucose transport into skeletal muscle cells. Among the two varieties, Njavara consistently exhibited superior antidiabetic potential, highlighting its value as a functional food and a promising natural source of antidiabetic bioactive compounds. These findings provide scientific support for the traditional medicinal use of these rice varieties and warrant further in vivo and clinical investigations to validate their therapeutic potential.

Chapter 1
Introduction

Diabetes mellitus is a chronic metabolic disorder that is increasing rapidly in both developing and industrialized countries. The two major forms of diabetes are Type 1 and Type 2 diabetes (American Diabetes Association, 2010; Jain & Saraf, 2010). Type 1 diabetes, commonly referred to as juvenile diabetes, is insulin-dependent and accounts for approximately 5% of all diabetes cases. Type 2 diabetes, which is non-insulin-dependent, generally occurs in adults over the age of 40 years. Chronic hyperglycemia associated with diabetes is strongly linked to long-term damage, dysfunction, and failure of various organs, particularly the eyes, kidneys, nerves, heart, and blood vessels (Huang et al., 2005). Diabetes adversely affects the metabolism of carbohydrates, proteins, and lipids, resulting in persistent hyperglycemia and abnormal lipid profiles. These metabolic disturbances can lead to several complications, including retinopathy, ketosis, polyuria, polyphagia, and cardiovascular diseases (Kumar & Clark, 2002).

Despite the widespread use of insulin and oral hypoglycemic agents, diabetes and its associated complications remain major global health concerns. The disease affects nearly 10% of the world's population and contributes significantly to morbidity and mortality (Burke et al., 2003). Furthermore, diabetes imposes a substantial economic burden on both individuals and nations, potentially hindering socioeconomic development (Mahabir & Gulliford, 1997). Several factors contribute to the onset of diabetes, including obesity, unhealthy dietary habits, sedentary lifestyle, genetic predisposition, insulin resistance, advancing age, and lifestyle changes associated with urbanization (Deepashree & Prakash, 2007). Consequently, effective management of diabetes remains a major global challenge.

Current treatment strategies include insulin therapy and various oral hypoglycemic drugs such as sulfonylureas, metformin, α-glucosidase inhibitors, and thiazolidinediones. However, these medications are often associated with adverse effects, including gastrointestinal disturbances, lactic acidosis, and hepatic complications (Rajalakshmi et al., 2009). According to Mentreddy et al. (2005), diabetes affected approximately 143 million people worldwide, and this number was projected to increase to 366 million by 2030 (Ponnusamy et al., 2011).

Medicinal plants have gained considerable attention as alternative therapeutic agents for diabetes management. More than 800 plant species have been reported to possess antidiabetic properties. Plants rich in bioactive compounds such as phenolics, flavonoids, alkaloids, terpenoids, coumarins, and glycosides exhibit significant biological activities, including antihyperglycemic effects (Grover et al., 2002). Interestingly, several conventional antidiabetic drugs have their origins in plant-derived compounds. Traditional herbal remedies are known to improve insulin sensitivity, stimulate insulin secretion, and reduce blood glucose levels. Many medicinal plants are widely used in traditional systems of medicine due to their efficacy and comparatively lower incidence of adverse effects. Their pharmacologically active constituents exert hypoglycemic effects through multiple mechanisms, including inhibition of α-amylase activity and modulation of various biochemical pathways involved in glucose metabolism (Murali Mohan, 2006).

Rice (*Oryza* spp.) is the staple food for more than half of the world's population and exhibits remarkable genetic diversity among the numerous varieties cultivated worldwide (Ito & Lacerda, 2019). Asia accounts for nearly 80% of global rice production, and approximately 90% of the Asian population depends on rice as a primary dietary component (Bhat & Riar, 2015; Chaudhari et al., 2018). Rice serves as an economical source of energy and is rich in both macro- and micronutrients (Ghanghas et al., 2022).

Among the genus Oryza, the two most important cultivated species are *Oryza sativa* L. and *Oryza glaberrima* Steud. *Oryza sativa* originated in Asia and was subsequently introduced to other parts of the world, whereas *O. laberrima* is indigenous to Africa and is cultivated exclusively there (Fageria & Baligar et al., 2003). In addition to its nutritional significance, rice has long been recognized for its medicinal value. Traditional Asian medical systems have used rice and rice-based preparations to treat various ailments. Reported therapeutic applications include anti-inflammatory effects on the respiratory tract, antidiabetic activity, nutritional supplementation, dermatological treatments, and management of gastrointestinal disorders and diarrhoea (Ahuja et al., 2008; Umadevi et al., 2012). Traditional Indian rice varieties possess extensive genetic diversity and are valued for their unique aroma, nutritional quality, and therapeutic properties. These traditional cultivars are rich sources of vitamin D, calcium, thiamine, riboflavin, glutamic acid, and dietary fibre (Bhat & Riar, 2015; Verma et al., 2018). They are also enriched with essential amino acids, antioxidants, phytochemicals, vitamin E, and other bioactive nutrients

(Devraj et al., 2020). Traditional rice varieties are considered beneficial for individuals with diabetes and hypertension due to their low fat and sugar content and the presence of bioactive compounds, such as γ-oryzanol, which is known to regulate lipid metabolism and reduce fat accumulation.

In addition to their nutritional value, traditional rice varieties contain several bioactive compounds present in trace amounts across different grain components, including the bran, germ, and endosperm (Moongngarm et al., 2012; Rondanelli et al., 2019). These bioactive constituents contribute to a range of health-promoting properties, including antioxidant, anti-inflammatory, and antidiabetic activities (Kariyawasam et al., 2016b). In Ayurveda, whole traditional rice grains are regarded not only as nutritious foods but also as medicinal substances that promote health and well-being (Rathna Priya et al., 2019). The health benefits of traditional rice are largely attributed to the presence of phytochemicals, phytonutrients, γ-oryzanol, tocols, neurotransmitters, and other biologically active compounds (Devraj et al., 2020).

Among the phytochemicals present in rice, phenolic acids constitute an important group. These compounds are characterized by a phenolic ring attached to an organic carboxylic acid and contribute significantly to the antioxidant potential of rice grains (Goufo et al., 2014; Sivamaruthi et al., 2018). Bound phenolic acids are particularly important because of their enhanced bioavailability and associated health benefits (Lum & Chong, 2012). Traditional rice varieties have been reported to contain higher levels of phenolic acids than many improved cultivars. Caffeic acid, for example, has been identified in certain traditional rice varieties but is absent in several modern improved varieties (Gunaratne et al., 2013).

Flavonoids represent another major class of phenolic compounds widely distributed in plants and recognized for their health-promoting properties (Babu & Liu, 2009; Bone & Mills, 2013). Several flavonoids, including quercetin, cirsimaritin, kaempferide, kaempferol, 5-hydroxy-3,3′,4′,7-tetramethoxyflavone, 3,3′,4′,7-tetramethoxyflavone, and flavone, have been identified in traditional rice varieties. These compounds are classified into different subclasses such as flavones, flavonols, and O-methylated flavonoids (Valarmathi et al., 2015). Owing to their antioxidant and biological activities, flavonoids contribute significantly to the functional properties of traditional rice.

Anthocyanins are another important group of bioactive compounds found predominantly in pigmented rice varieties. These water-soluble pigments are responsible for the purple, blue, red, and black coloration observed in rice grains and other cereal crops (Limtrakulet al., 2020; Mbanjo et al., 2020). Several studies have reported that pigmented rice varieties contain significantly higher anthocyanin concentrations than non-pigmented varieties (Nayeem et al., 2021; Pathak et al., 2017; Saikia et al., 2012). Due to their strong antioxidant activity, anthocyanins have attracted considerable attention for their potential to prevent chronic diseases and promote human health. In recent years, bioactive compounds in rice, including phenolics, flavonoids, carotenoids, phytosterols, and related phytochemicals, have attracted increasing scientific interest due to their potential health benefits (Jaime & Santoyo, 2021). Although these compounds occur in relatively small quantities, they play important roles in disease prevention and health promotion. Epidemiological studies have demonstrated that regular consumption of foods rich in bioactive compounds may reduce the risk of several chronic disorders, including diabetes, cardiovascular diseases, cancer, cataracts, Alzheimer's disease, and age-related degenerative conditions (Siriwardhana et al., 2013). Furthermore, recent studies suggest that certain bioactive compounds may enhance immune function and help protect against viral infections (Azam et al., 2023).

Although the basic elemental composition of rice remains relatively similar across different varieties (Verma & Srivastav, 2020), traditional rice cultivars generally contain higher concentrations of beneficial phytochemicals than modern high-yielding varieties (Devraj et al., 2020). These phytochemicals include anthocyanins, proanthocyanidins, γ-oryzanol, phytic acid, and various phenolic compounds, including phenolic acids, flavonoids, and tannins. Collectively, these compounds exhibit strong antioxidant and free-radical scavenging activities, thereby providing numerous health benefits (Nadini Thushara et al., 2019). Most phytochemicals are concentrated in the bran and pericarp layers of the rice grain (Rathna Priya et al., 2019). During parboiling, however, some of these compounds migrate from the outer layers into the starchy endosperm, thereby improving the nutritional quality of the milled grain (Lee, 2017).

Black rice is particularly rich in anthocyanins, a group of bioactive flavonoid pigments responsible for its characteristic dark coloration (Agustinet al., 2021; Maisuthisakul & Changchub, 2014; Rajendran et al., 2018; Wongsa et al., 2018). Several anthocyanin compounds, including quercetin-3-O-galactoside, 5-pyranopelargonidin-3-O-glucoside, cyanidin-3-O-rutinoside, pelargonidin-3-

O-diglucoside, and cyanidin-3-O-galactoside, have been identified in pigmented traditional rice varieties. Among these, cyanidin-3-glucoside is recognized as the predominant anthocyanin present in rice grains (Bhat & Riar, 2017). Traditional black rice has also been reported to contain pelargonidin-3-O-glucoside (Agustin et al., 2021). Cyanidin-3-glucoside exhibits antidiabetic activity by inhibiting α-amylase, thereby regulating postprandial blood glucose levels (Sui et al., 2016). Consequently, anthocyanins are considered key contributors to the differences in phytochemical composition and antioxidant potential observed between colored and non-colored rice varieties (Devraj et al., 2020).

Chak-Hao Poreiton, a traditional black rice variety from India, is known for its high anthocyanin content and has been reported to possess both probiotic and antidiabetic properties. Anthocyanins isolated from this rice variety have been shown to interact with glucose transporter-1 (GLUT1) and key carbohydrate-metabolizing enzymes such as pancreatic α-amylase and β-glucosidase, suggesting a role in glucose homeostasis (Rajendran & Chandran, 2020). Anthocyanins are also present in appreciable quantities in red rice varieties (Agustin et al., 2021; Maisuthisakul & Changchub, 2014; Rajendran et al., 2018; Wongsa et al., 2018). The anthocyanins present in both red and black rice exhibit strong antioxidant activity and contribute significantly to their health-promoting properties (Agustin et al., 2021). Interestingly, measurable amounts of anthocyanins have also been detected in certain white rice cultivars, although their concentrations are considerably lower than those found in pigmented varieties (Agustin et al., 2021; Devraj et al., 2020; Nayeem et al., 2021; Pathak et al., 2017; Rajendran et al., 2018).

Environmental conditions also influence anthocyanin accumulation in rice grains. Jaksomsak et al. (2021) reported that anthocyanin concentrations were lower in aerobically cultivated purple rice varieties than in those grown under wetland conditions. Therefore, traditional wetland cultivation practices may enhance the nutritional and health-promoting properties of pigmented rice varieties. Milling and polishing significantly reduce the concentration of bioactive compounds and antioxidant capacity in rice grains. These processing operations remove the bran layer, which serves as the principal reservoir of phenolics, flavonoids, anthocyanins, and other phytochemicals. Consequently, polished rice generally exhibits lower antioxidant activity than unpolished rice (Mannan et al., 2013; Mudoi & Das, 2019; Thennakoon & Ekanayake, 2021). Numerous studies have demonstrated that the antioxidant activity of colored rice varieties is primarily attributed to

their high concentrations of anthocyanins, phenolic compounds, and flavonoids (Agustin et al., 2021; Ashokkumar et al., 2020; Bhat & Riar, 2017; Chanu et al., 2016; Maisuthisakul & Changchub, 2014; Mannan et al., 2013; Pathak et al., 2017; Rajendran et al., 2018; Saikia et al., 2012; Shin et al., 2016). However, some non-pigmented indigenous rice varieties, such as Vandana, have also been reported to possess appreciable antioxidant activity, suggesting that compounds other than anthocyanins may contribute to their antioxidant potential (Pathak et al., 2017). Consumption of pigmented rice has been shown to enhance antioxidant status and reduce plasma malondialdehyde levels, an indicator of oxidative stress, thereby promoting overall health.

Among the traditional rice varieties of Kerala, Njavara and Rakthashali are recognized as important medicinal rice cultivars with considerable therapeutic value. Njavara rice is extensively used in Ayurvedic preparations such as Njavara Kizhi and is employed in the treatment of arthritis, cervical spondylosis, muscular atrophy, skin disorders, neurological ailments, and rejuvenation therapies. Rakthashali, a red-pigmented rice variety, is rich in phenolic compounds, flavonoids, anthocyanins, and other bioactive constituents that contribute to its medicinal properties.

Njavara belongs to the species *Oryza sativa* L. and is considered one of the oldest and most genetically distinct rice varieties cultivated in India (Nair & Thomas, 2001). Traditionally cultivated only in Kerala, South India, Njavara is primarily grown for medicinal purposes and occupies a unique place in Ayurvedic medicine. It is generally cultivated as an upland crop under conditions of limited water availability (Menon, 2004). Although its yield potential is relatively low and its pest resistance is moderate, Njavara is still conserved for its exceptional medicinal value. Morphologically, Njavara resembles common rice varieties, but the husk colour varies from golden yellow to brownish-black depending on environmental and edaphic factors (Menon, 2004). Commonly referred to as the “Sashtika” type in Ayurvedic literature, Njavara matures within approximately 60 days under semi-dry cultivation conditions.

Despite its medicinal importance, Njavara faces the risk of genetic erosion due to its low productivity, high cultivation costs, and limited commercial utilization outside Ayurvedic applications. Ayurveda considers Njavara a unique therapeutic rice variety, beneficial for maintaining the health of the respiratory, digestive, and circulatory systems (Deepa et al., 2008). It is widely used as a functional food and in the management of neurological disorders, rheumatoid arthritis, paralysis, and rejuvenation therapies.

Phytochemical investigations of Njavara Black rice bran have revealed the presence of tricin and two rare flavonolignans, namely tricin 4′-O-(erythro-β-guaiacylglyceryl) ether and tricin 4′-O-(threo-β-guaiacylglyceryl) ether. Comparative HPLC analysis demonstrated that tricin levels in Njavara Black were approximately 39.64-fold and 16.12-fold higher than those found in the non-medicinal rice varieties Sujatha and Palakkadan Matta, respectively. This study also reported these flavonolignans for the first time in *Oryza sativa*. Biological evaluation further revealed that tricin and the threo-form of flavonolignan exhibited more than 65% anti-inflammatory activity in carrageenan-induced paw edema in rats at a dose of 2 mg kg^{-1} after 5 hours. These findings provide scientific evidence supporting the traditional medicinal use of Njavara and justify its preference over common rice varieties in indigenous healthcare systems (Mohanlal et al., 2011).

Rakthashali is a traditional red rice variety native to Kerala, renowned for its exceptional nutritional and medicinal properties. The grains are rich in proteins, minerals, and various bioactive compounds that contribute to their health-promoting effects. Rakthashali rice has been reported to contain approximately 5.69 mg of calcium and 1.67 mg of iron per 100 g of grain (Girijan et al., 2023). Studies have demonstrated that both Rakthashali rice flour and bran possess significant antioxidant and cytotoxic activities. Furthermore, extracts from this variety exhibited antiproliferative effects against the MCF-7 human breast cancer cell line, indicating its potential role in cancer prevention and management. These findings suggest that Rakthashali rice may serve as an important dietary source of natural antioxidants and could help prevent various lifestyle-related diseases (Girijan et al., 2023).

Traditionally, Rakthashali has been valued in Ayurveda for its therapeutic properties. It is believed to enhance fertility, improve vision and skin health, and aid in the treatment of ailments such as fever, ulcers, and hemorrhoids (Bhat & Riar, 2015). In addition, traditional medicinal literature associates Rakthashali consumption with several health-promoting effects, including alleviation of thirst, enhancement of vitality, and restoration of physiological balance (Kotur &Gadad, 2025). The grains are also rich in nutritionally important minerals such as calcium, zinc, and iron, further supporting their functional food value (Sha et al.,2025).

Pigmented rice varieties, including Rakthashali, derive their characteristic coloration primarily from anthocyanins and proanthocyanidins concentrated in the bran layer. These compounds are recognized for their potent antioxidant activity and numerous health benefits. Boue et al. (2016)

evaluated the phenolic, flavonoid, anthocyanin, and proanthocyanidin contents of several rice bran extracts obtained from brown, red, and purple rice varieties. The study further assessed the inhibitory effects of these extracts on α-amylase and α-glucosidase, two key enzymes involved in carbohydrate digestion. The results revealed that red rice bran extracts significantly inhibited α-amylase activity, while both red and purple rice bran extracts effectively inhibited α-glucosidase activity. These findings highlight the potential role of pigmented rice varieties in the dietary management of diabetes by modulating carbohydrate metabolism. Coloured rice varieties, including red, black, and purple rice, contain a diverse array of bioactive compounds, including phenolic acids, flavonoids, anthocyanins, and proanthocyanidins. These phytochemicals are largely responsible for the functional and therapeutic properties of pigmented rice. Increasing consumer interest in colored rice is attributed to its superior nutritional value and the presence of health-promoting bioactive compounds. Several studies have demonstrated that these phytochemicals exhibit antioxidant, anti-inflammatory, anticancer, and antidiabetic activities, thereby enhancing the functional food potential of pigmented rice varieties (Wongsa, 2020).

Despite extensive documentation of the nutritional and medicinal significance of traditional rice varieties, scientific evidence regarding their direct effects on cellular glucose metabolism remains limited. Njavara and Rakthashali, two important medicinal rice varieties of Kerala, are particularly rich in phenolic compounds, flavonoids, anthocyanins, and other bioactive constituents with established antioxidant and anti-inflammatory properties. Although previous studies have highlighted the therapeutic potential of pigmented rice and its role in diabetes management, the cellular mechanisms underlying these effects remain scarce. Therefore, the present study was undertaken to evaluate the antidiabetic potential of Njavara and Rakthashali rice extracts using *in vitro* cell culture models. The study employed L6 skeletal muscle cells and HepG2 hepatic cells to assess cytotoxicity and glucose uptake activity using MTT and glucose uptake assays, respectively, as well as alpha amylase activity. The findings are expected to provide scientific evidence supporting the traditional use of these medicinal rice varieties and contribute to the development of functional foods for diabetes management.

Chapter 2
Review of Literature

2.1 Diabetes Mellitus and the Nutritional Significance of Medicinal Rice Varieties

Diabetes mellitus is a chronic metabolic disorder that is increasing rapidly in both developing and industrialized nations. The disease is broadly classified into two major types: type 1 diabetes mellitus and type 2 diabetes mellitus (Jain & Saraf, 2010; American Diabetes Association, 2010). Type 2 diabetes mellitus (T2DM), also known as non-insulin-dependent diabetes mellitus, is characterized by impaired insulin secretion, insulin resistance, or both, leading to chronic hyperglycemia and associated metabolic abnormalities Approximately 90–95% of all diabetes cases are attributed to T2DM (Li et al., 2004). If left untreated, diabetes can result in severe complications such as cardiovascular diseases, stroke, blindness, kidney failure, and neuropathy (Boue et al.,2016) . The increasing prevalence of chronic lifestyle diseases has spurred interest in functional foods, which provide health benefits beyond basic nutrition. Functional foods have gained considerable popularity among health-conscious consumers because of their potential role in the prevention and management of chronic diseases (Padmavathi, 2013; Bigliardi & Galati, 2013). Rice (*Oryza sativa* L.) is one of the most important cereal crops worldwide and serves as a staple food for more than half of the global population. It is cultivated in over 100 countries and plays a crucial role in global food security (Nagendra et al., 2011; Sompong et al., 2011). Since rice is a major dietary component in many populations, identifying rice cultivars with antidiabetic properties is important for the prevention and management of diabetes and its associated complications (Bhonsle et al., 2010). In recent years, considerable attention has been focused on plant-derived bioactive compounds because of their ability to regulate glucose metabolism, enhance insulin sensitivity, and reduce oxidative stress.

2.2 Medicinal Rice Varieties of Kerala

Kerala possesses several indigenous rice landraces that have traditionally been used in Ayurvedic medicine. Among these, Njavara and Rakthashali are historically important medicinal rice varieties recognized for their therapeutic value. These rice varieties are rich in minerals, antioxidants, dietary fiber, and various bioactive compounds that may contribute to their health-

promoting properties. Several studies have reported that traditional rice varieties possess superior nutritional qualities compared to commonly consumed polished white rice varieties.

2.3 Nutritional and Antidiabetic Significance of Rice Varieties

Hu et al. (2012) conducted a meta-analysis of prospective cohort studies to examine the dose-response relationship between white rice consumption and the risk of type 2 diabetes mellitus. The study demonstrated that higher consumption of white rice was associated with a significantly increased risk of T2DM, particularly among Asian populations, including Chinese and Japanese communities.

According to Rathna Priya et al. (2019), rice is a major staple food and one of the most important cereal crops in developing countries. India is the second-largest producer of rice after China. Although more than 40,000 rice varieties are known worldwide, only a limited number are currently cultivated and consumed extensively. Besides conventional white rice, several speciality rice varieties, including colored and aromatic rice, are also cultivated. The authors reported that specialty rice varieties possess superior nutritional profiles compared to conventional white rice. Colored rice varieties, which derive their pigmentation primarily from anthocyanins accumulated in the bran layer, are particularly rich in antioxidants and phytochemicals. Furthermore, Rathna Priya et al. (2019) highlighted that rice bran, a by-product of the rice milling industry, remains underutilized despite being a rich source of dietary fiber and bioactive compounds. Rice bran has significant potential for the development of functional foods and value-added products. The authors concluded that increased utilization and conservation of speciality rice varieties and their by-products could contribute substantially to nutritional security while preventing the loss of valuable genetic resources.

Goufo and Trindade (2014) reported that the antioxidant compounds present in rice may contribute to the lower incidence of several chronic diseases observed in populations with high rice consumption. The authors compiled data from 316 scientific publications to establish a comprehensive database of rice antioxidant compounds, including phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ-oryzanol, and phytic acid. Their analysis revealed that grain color significantly influences antioxidant activity. Black rice varieties exhibited the highest antioxidant activity, followed by red, purple, and brown rice varieties. The

study further demonstrated that most phenolic acids and proanthocyanidins occur predominantly in insoluble forms, whereas flavonoids and anthocyanins are mainly present in soluble forms.

2.4 Njavara Rice: Phytochemical and Medicinal Properties

Deepa et al. (2008) investigated the physicochemical characteristics and nutritional composition of Njavara, a traditional medicinal rice variety of India, to better understand its therapeutic potential. The study compared the nutritional and physicochemical properties of dehusked Njavara rice with two commonly cultivated non-medicinal rice varieties, Jyothi (red rice) and IR 64 (brown rice). Njavara rice contained 1628 kJ of energy per 100 g, along with 73% carbohydrates, 9.5% protein, 2.5% fat, and 1.4% ash. The carbohydrate, fat, triglyceride, fatty acid profile, and apparent amylose content of Njavara were comparable to those of Jyothi and IR 64. However, Njavara exhibited significantly higher protein content (16.5%) and elevated levels of B-complex vitamins, including thiamine (27–32%), riboflavin (4–25%), and niacin (2–36%), compared to the other two varieties. The total dietary fiber content of Njavara was found to be 34–44% higher than that of Jyothi and IR 64. In addition, Njavara contained higher concentrations of essential minerals, including phosphorus, potassium, magnesium, sodium, and calcium. The cooking time of dehusked Njavara rice was approximately 38 minutes, compared to 30 minutes for Jyothi and IR 64. The cooked Njavara grains exhibited a characteristic slimy texture, attributed to the presence of non-starch polysaccharides.

Simi and Abraham (2008) evaluated the physicochemical, rheological, and thermal properties of Njavara rice starch and compared them with those of native Chamba rice starch. The study revealed that Njavara starch possessed higher swelling power, solubility, water absorption capacity, and gelatinization temperature than native rice starch. Furthermore, Njavara starch exhibited a significantly higher enthalpy of gelatinization and degraded at higher temperatures, indicating superior thermal stability. Pasting property analysis demonstrated higher peak viscosity, breakdown viscosity, and setback values, suggesting favorable processing characteristics for food products requiring high-temperature treatments. These thermal attributes may contribute to the traditional therapeutic applications of Njavara rice, particularly in Ayurvedic heat-based treatments. Rheological studies further showed that Njavara starch gelatinized at a higher temperature (68°C) than native rice starch (58°C). The starch also exhibited greater gel strength,

as evidenced by the larger difference between storage modulus (G′) and loss modulus (G″) values. Moreover, the higher torque and lower tan δ values observed for Njavara starch indicated the formation of a stronger, more stable gel structure than native rice starch.

Mohanlal et al. (2012) investigated the phytochemical composition of Njavara rice bran and isolated three bioactive compounds: tricin, tricin 4′-(erythro-β-guaiacylglyceryl) ether, and tricin 4′-O-(threo-guaiacylglyceryl) ether. Notably, the latter two compounds were rare flavonolignans identified in rice bran for the first time. The anti-inflammatory activities of these compounds were subsequently evaluated in vivo. The results demonstrated that Njavara rice contains higher concentrations of tricin, a multifunctional bioactive flavonoid with promising nutraceutical properties, compared to commonly cultivated staple rice varieties. Additionally, the presence of rare flavonolignans with potent antioxidant and anti-inflammatory activities further enhanced the medicinal value of Njavara. These findings provide scientific support for the long-standing preference for Njavara rice in Ayurvedic medicine and highlight its potential as a functional food with significant health-promoting properties.

2.5 Rakthashali Rice: Phytochemical and Medicinal Properties

Rakthashali is a traditional medicinal red rice variety widely recognized in Ayurveda for its therapeutic and nutritional significance. As a pigmented rice cultivar, Rakthashali is characterized by the presence of anthocyanins, phenolic compounds, and flavonoids in the bran layer, which impart the characteristic red coloration to the grains. Due to their rich phytochemical composition, pigmented rice varieties have attracted considerable scientific attention for their superior antioxidant activity and health-promoting properties compared to polished white rice. Sompong et al. (2011) evaluated the physicochemical and antioxidative properties of red and black rice varieties from Thailand, China, and Sri Lanka. The study demonstrated that colored rice varieties possessed significantly higher total phenolic content and antioxidant activity than white rice varieties. These findings highlight the potential of pigmented rice as a valuable source of natural antioxidants that may help prevent oxidative stress-related diseases.

In recent years, the nutraceutical potential of traditional rice landraces has attracted increasing attention, with evidence suggesting that consuming pigmented rice is associated with a reduced risk of chronic diseases. Mikhina et al. (2026) investigated the bioactive compounds in six traditional rice landraces, including three red-pigmented varieties (Rakthashali, Chennellu, and

Njavara), two white varieties (Kala Namak and Chomala), and one black variety (Black Jasmine). The study also included 'Jyothi', an important red rice variety released by Kerala Agricultural University, as a reference cultivar. Among the varieties analyzed, Black Jasmine exhibited the highest concentrations of iron and zinc (19.17 and 39.47 mg 100 g^{-1}, respectively), anthocyanins (188.21 mg 100 g^{-1}), flavonoids (147.30 mg 100 g^{-1}), antioxidant activity (IC_{50} = 7.40), and in vitro digestibility (59%). However, several traditional rice landraces exhibited higher levels of specific nutraceutical compounds than the reference variety, Jyothi. Notably, Rakthashali recorded the highest γ-oryzanol content (72.86 mg 100 g^{-1}) and the lowest moisture content (11.19%) among the varieties studied.

High-performance liquid chromatography (HPLC) analysis further revealed that Rakthashali contained substantial amounts of bioactive phenolic compounds, including catechin, syringic acid, caffeic acid, vanillic acid, chlorogenic acid, kaempferol, and epicatechin. These phytochemicals are known for their antioxidant, anti-inflammatory, and antidiabetic activities. Based on the phytochemical profile, the authors identified Rakthashali as one of the most promising medicinal rice varieties among the landraces evaluated. To explore the therapeutic potential of these compounds, the identified phenolics were subjected to molecular docking studies against three important protein targets associated with lifestyle diseases: cyclooxygenase-2 (COX-2), dipeptidyl peptidase-IV (DPP-IV), and nuclear factor-kappa B (NF-κB). The results indicated that chlorogenic acid exhibited the strongest binding affinity toward all three target proteins, suggesting its potential role in mitigating inflammation, diabetes, and other chronic metabolic disorders. The study emphasized the importance of conserving pigmented traditional rice landraces, not only for preserving their valuable genetic resources but also as a rich source of health-promoting phytonutrients.

The antidiabetic potential of flavonoids, abundant in coloured rice varieties such as Rakthashali, has also been demonstrated by Tadera et al. (2006). The authors investigated the inhibitory effects of various flavonoids on α-amylase and α-glucosidase, two key enzymes involved in carbohydrate digestion. Their results showed that several flavonoids effectively inhibited the activity of these enzymes, thereby slowing carbohydrate hydrolysis and glucose absorption. Such enzyme inhibition may contribute to improved postprandial glycemic control and supports the potential use of flavonoid-rich rice varieties in diabetes management. Therefore, the high concentrations of

phenolic compounds and flavonoids present in Rakthashali may contribute significantly to its antidiabetic and health-promoting properties.

2.6 Antidiabetic Activity of Pigmented Rice Varieties

Pigmented rice varieties have attracted considerable scientific interest due to their high levels of bioactive compounds, including anthocyanins, proanthocyanidins, flavonoids, and phenolic acids, which possess antioxidant and antidiabetic properties. These compounds are predominantly concentrated in the bran layer of the grain and have been associated with improved glucose metabolism, enhanced insulin sensitivity, and reduced oxidative stress.

Boue et al. (2016) investigated the antidiabetic potential of colored rice bran extracts by analyzing five rice bran samples, including two red, two purple, and one brown rice variety. The study quantified the phenolic, flavonoid, anthocyanin, and proanthocyanidin contents of each extract and evaluated their ability to inhibit α-amylase and α-glucosidase, two key enzymes involved in starch digestion. The results revealed that all red and purple rice bran extracts effectively inhibited α-glucosidase activity, whereas inhibition of α-amylase activity was observed only in red rice bran extracts. These findings suggest that bioactive compounds in pigmented rice may help regulate postprandial blood glucose levels by modulating carbohydrate-digesting enzymes.

The authors also assessed the effects of rice bran extracts on glucose uptake in 3T3-L1 adipocytes, an important mechanism that maintains glucose homeostasis. Treatment with red rice bran extracts increased basal glucose uptake by 2.3–2.7 fold, while purple rice bran extracts enhanced glucose uptake by 1.9–3.1 fold. Among the tested samples, the proanthocyanidin-rich fraction of red rice bran exhibited the strongest inhibitory effects on digestive enzymes and the greatest stimulation of glucose uptake. Furthermore, both red and purple rice bran extracts upregulated the expression of GLUT1 and GLUT4 genes, as well as genes involved in the insulin-signaling pathway, indicating their potential role in improving insulin sensitivity and glucose utilization (Boue et al., 2016).

Oxidative stress is recognized as a major contributor to the development and progression of diabetes mellitus and its associated complications. Chronic hyperglycemia promotes the excessive production of reactive oxygen species (ROS), leading to cellular damage and metabolic dysfunction. Ceriello and Motz (2004) highlighted the critical role of oxidative stress in the

pathogenesis of insulin resistance, diabetes mellitus, and cardiovascular diseases. The authors suggested that dietary intake of antioxidant-rich foods may reduce oxidative stress and improve metabolic abnormalities associated with diabetes.

Anthocyanins, a major bioactive compound found in pigmented rice varieties, have been extensively studied for their beneficial effects on glucose metabolism. Tsuda (2012) reviewed the physiological functions of anthocyanins and reported that anthocyanin-rich foods improve insulin sensitivity and glucose homeostasis by enhancing insulin-signaling pathways and regulating glucose transport mechanisms. Experimental studies in diabetic animal models have demonstrated that anthocyanins can reduce hyperglycemia, alleviate oxidative stress, and improve overall metabolic health. Since Rakthashali is a red-pigmented rice variety rich in anthocyanins, these compounds may significantly contribute to its potential antidiabetic properties.

Min et al. (2012) examined the polyphenolic composition and antioxidant activities of whole-grain rice varieties possessing different bran colors. The study characterized both solvent-extractable (free) and cell wall-bound fractions of phenolic compounds, flavonoids, anthocyanins, and proanthocyanidins. The results demonstrated that red and purple bran rice varieties contained significantly higher levels of total phenolics, flavonoids, and antioxidant activity compared with light-colored bran rice and other cereal grains ($P< 0.05$). These enhanced antioxidant capacities were primarily attributed to the elevated concentrations of anthocyanins and proanthocyanidins in pigmented rice varieties.

The authors further observed that bound phenolic and flavonoid fractions constituted approximately half of the total phenolic and flavonoid content in light-colored bran rice, whereas their relative contribution was lower in red and purple bran rice varieties ($P< 0.05$). Strong positive correlations were observed between total phenolic content and antioxidant capacity across three assays. These findings provide further evidence that pigmented rice varieties possess superior antioxidant potential and may offer significant health benefits, particularly in the prevention and management of oxidative stress-related disorders such as diabetes mellitus.

2.7 Bioactive Compounds and Antidiabetic Potential of Pigmented Rice

Min et al. (2012) further characterized the polyphenolic composition of pigmented rice varieties and reported that red bran rice contained a proanthocyanidin concentration of 1.27 mg g^{-1}. The

proanthocyanidins were distributed as 6.5% monomers to trimers (1–3 mers), 33.5% oligomers (4–6 mers), 30.6% polymers of intermediate size (7–10 mers), and 29.4% high-molecular-weight polymers (>10mers). Among the anthocyanins identified, cyanidin-3-glucoside was the predominant compound, followed by peonidin-3-glucoside. The study also revealed considerable variation in anthocyanin composition among different purple rice cultivars. These findings demonstrated that whole-grain rice varieties with different bran colors contain distinct polyphenolic profiles, which may contribute to their diverse biological activities and potential health benefits.

Shalini et al. (2012) investigated the anti-inflammatory properties of tricin, a bioactive flavonoid abundantly present in the medicinal rice variety Njavara. Flavonoids are naturally occurring phytochemicals widely recognized for their ability to modulate both acute and chronic inflammatory responses. Phytochemical analyses revealed that Njavara contains substantially higher concentrations of tricin than commonly cultivated rice varieties, supporting its long-standing use in traditional medicine. To elucidate the molecular basis of its therapeutic effects, the authors evaluated the role of tricin in regulating inflammatory mediators produced by lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells. Treatment with tricin significantly suppressed the production of major pro-inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2), and nitric oxide (NO). Furthermore, tricin inhibited the expression of cyclooxygenase enzymes, matrix metalloproteinases, and nitric oxide synthase isoforms. The study also demonstrated modulation of the nuclear factor-kappa B (NF-κB) signaling pathway, as evidenced by altered nuclear translocation of the p65 subunit. These findings suggest that tricin exerts its anti-inflammatory effects by regulating multiple cell-signaling pathways and inflammatory mediators. Given the established relationship between chronic inflammation and insulin resistance, tricin's anti-inflammatory activity may also contribute to the antidiabetic potential of medicinal rice varieties such as Njavara.

Munkong et al. (2022) evaluated the effects of red rice bran extract (RRBE) on insulin resistance induced by a high-fat diet in experimental mice. The study demonstrated that RRBE supplementation significantly improved glucose-insulin homeostasis in insulin-resistant animals. The beneficial effects were associated with the modulation of genes involved in insulin

metabolism, including insulin and insulin-degrading enzyme (IDE), as well as genes associated with insulin signaling and glucose transport, such as IRS1, IRS2, GLUT4, and GLUT2. These molecular changes enhanced insulin sensitivity and glucose utilization, suggesting that bioactive compounds present in red rice bran may play an important role in the prevention and management of type 2 diabetes mellitus.

2.8 In Vitro Cell-Based Studies on Antidiabetic Activity of Medicinal Plants and Rice Bioactive compounds

Anandharajan et al. (2006) investigated the antidiabetic potential of *Syzygiumcumini* seed extract using L6 skeletal muscle cells. The study demonstrated that treatment with the extract significantly enhanced glucose uptake in L6 myotubes without causing cytotoxic effects. The increased glucose utilization was attributed to improved GLUT4 translocation and insulin-mimetic activity. The authors concluded that *S. cumini* possesses promising antidiabetic properties and may serve as a potential source of natural antidiabetic agents. Patel et al. (2012) evaluated the effects of several medicinal plant extracts on glucose uptake in L6 myotubes. Their findings showed that flavonoid-rich extracts significantly increased glucose uptake, comparable to insulin-treated controls. The study suggested that phytochemicals such as flavonoids and phenolic compounds could stimulate glucose transport pathways in skeletal muscle cells and thereby contribute to glycemic control.

Lee et al. (2006) investigated the effects of berberine, a plant-derived isoquinoline alkaloid, in insulin-resistant L6 myotubes. Berberine treatment significantly increased glucose uptake by activating AMP-activated protein kinase (AMPK) and enhancing GLUT4-mediated glucose transport. The study highlighted the usefulness of L6 cells as an in vitro model for screening antidiabetic compounds. Yan et al. (2016) examined the antidiabetic activity of anthocyanin-rich plant extracts using HepG2 cells. The study reported that anthocyanins significantly improved glucose consumption and reduced intracellular oxidative stress in insulin-resistant HepG2 cells. The authors suggested that anthocyanins may improve hepatic glucose metabolism through activation of the P13K/AKT signaling pathway. Boue et al. (2016) evaluated coloured rice bran extracts, including those from red and purple rice varieties, for their antidiabetic effects. Using 3T3-L1 adipocytes, the study demonstrated enhanced glucose uptake and increased expression of GLUT1 and GLUT4 genes following treatment with pigmented rice bran extracts. The proanthocyanidin-rich fraction of red rice bran showed the strongest glucose uptake activity and

enzyme inhibitory effects. These findings indicate that bioactive compounds present in pigmented rice may improve glucose homeostasis and insulin sensitivity.

These studies collectively demonstrate that L6 skeletal muscle cells and HepG2 hepatic cells are effective in vitro models for evaluating the antidiabetic potential of plant-derived bioactive compounds. They also provide evidence that phenolics, flavonoids, anthocyanins, and proanthocyanidins present in medicinal plants and pigmented rice varieties can enhance glucose uptake, improve insulin sensitivity, and modulate key molecular pathways involved in glucose metabolism.

Chapter 3
Aim and Objectives

Aim

To evaluate the antidiabetic potential of the medicinal rice varieties Njavara and Rakthashali through in vitro assessment of α-amylase inhibitory activity, cytotoxicity, glucose uptake, and GLUT4 gene expression using L6 skeletal muscle and HepG2 hepatic cell lines.

Objectives

1. To prepare methanolic extracts of the medicinal rice varieties Njavara and Rakthashali for in vitro antidiabetic evaluation.
2. To evaluate the α-amylase inhibitory activity of Njavara and Rakthashali rice extracts as an indicator of their potential to regulate carbohydrate digestion.
3. To determine the cytotoxicity and glucose uptake potential of the rice extracts in L6 skeletal muscle cells and HepG2 hepatic cells using the MTT assay and glucose uptake assay.
4. To analyse the effect of Njavara and Rakthashali rice extracts on GLUT4 gene expression in treated and untreated L6 skeletal muscle cells using qRT-PCR.
5. To compare the overall antidiabetic potential of Njavara and Rakthashali rice varieties based on α-amylase inhibitory activity, cytotoxicity, glucose uptake, and GLUT4 gene expression.

Chapter 4

Materials and Methods

4.1 Materials

4.1.1 Rice Varieties Selected for the Study

Two medicinal traditional rice landraces, Njavara (black) and Rakthashali, were selected for the present study. Njavara (black) seeds were procured from Njavara Ecofarm, Chittoor, Palakkad, Kerala, while Rakthashali seeds were collected from a farmer's field in Chirayinkil, Thiruvananthapuram, Kerala. Both rice varieties are recognized for their medicinal significance and were used for the preparation of methanolic extracts for subsequent in vitro antidiabetic investigations.Morphology of the selected rice varieties were shown in Figure 1.



Figure 1: Grain morphology of the rice varieties used in the study

4.1.2 Cell lines used

- L6 Skeletal Muscle cell line
- HepG2 Human Carcinoma cell line

4.1.3 Laboratory Materials

- Micropipettes and sterile tips
- T-25 and T-75 cell culture flasks
- 6-well culture plates
- 24-well plates

- 96-well microplates
- Eppendorf tubes, PCR tubes
- Whatman filter paper

4.2 Reagents

4.2.1 Reagents for rice extract preparation

- Methanol
- Liquid nitrogen

4.2.2 Reagents for Alpha amylase inhibition assay

- Alpha amylase enzyme
- Starch substrate solution
- Sodium phosphate buffer (pH 6.9)
- 3,5-Dintrosalicylic acid (DNSA) reagent
- Sodium potassium tartrate solution
- Metformin (reference standard,10 mg/ml)
- Distilled water

4.2.3 Reagents for Cytotoxic Screening

- Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS)
- antibiotic solution (1%)
- Phosphate Buffered Saline (PBS)
- Dimethyl sulfoxide (DMSO)
- MTT reagent (5 mg/mL in PBS)
- Detergent (50 % DMF + 20 % SDS).
- Doxorubicin (standard drug, MW: 579.98 g/mol)

4.2.4 Reagents for Glucose Uptake Assay

- ab136955 Glucose uptake assay kit (colorimetric) (Abcam, USA)

Glucose uptake assay kit components:-

- Assay buffer 3
- Extraction buffer I
- Neutralization buffer II
- Enzyme mix III
- Glutathione reductase
- Recycling mix
- DTNB(5,5' –Dithiobis-(2-nitrobenzoic acid)
- 2 Deoxyglucose(2-DG)
- 2 DG6p standard

Krebs-Ringer-Phosphate-Hepes (KRPH) buffer (pH 7.4) composition

- 20 mM HEPES
- 5mM KH_2PO_4
- 1 mM $MgSO_4$
- 1 mM $CaCl_2$
- 136 mM NaCl
- 4.7 mM KCl
- 2% bovine serumalbumin

4.2.5 Reagents for RNA isolation

- TRIzol reagent (Invitrogen, USA)
- Chloroform
- Ethanol (75%)
- DEPC-treated water
- Nuclease-free water

4.2.6 cDNA Synthesis and QRT-PCR Reagents

- iScript cDNA Synthesis Kit (Bio-Rad, USA)
- SYBR® Green Master mix (Bio-Rad, USA)
- Forward and reverse primers (Glut 4 and GAPDH)

4.3 Instruments

- 4˚C Refrigerator
- Vortex mixer
- Orbital shaker
- Rotor evaporator
- Sonicator
- UV–Visible spectrophotometer (Thermo Scientific, USA)
- Centrifuge (Eppendorf, Germany)
- Analytical weighing balance (Shimadzu, Japan)
- Hot air oven (Thermo Fischer Scientific, USA)
- Magnetic stirrer (Bionics Scientific)
- pH meter (Grant instruments, UK)
- Autoclave (Bionics Scientific)
- Water bath (Grant instruments, UK)
- Gel electrophoresis apparatus (Bio-Rad, USA)
- Hemocytometer (Thermo Fischer Scientific)
- CO_2 incubator (Thermo Fischer Scientific)
- Microplate reader (BioTek 800 TS, USA)

4.4 Methods

4.4.1 Preparation of Methanolic Rice Extract

The dehusked Njavara and Rakthashali rice varieties were finely powdered using a sterile mortar and pestle for extraction. Approximately 30 g of each powdered rice sample was weighed separately into two conical flasks. To each flask, 50 mL of methanol was added, and the contents were mixed thoroughly using a vortex mixer. The flasks were tightly sealed and incubated at 37°C in an orbital shaker for 14 days.After incubation, the extracts were concentrated using a rotary evaporator to remove methanol and obtain crude extracts. The semisolid extracts were subsequently lyophilized to obtain powdered extracts. The dried samples were collected, properly sealed, and stored at 4°C until further use.

For experimental studies, 10 mg of each powdered extract was dissolved in 1 mL of DMSO in sterile McCartney tubes. The tubes were tightly sealed and sonicated at 45–50°C for five cycles of 20 minutes each to ensure complete dissolution. The prepared extracts were stored at 4°C and used for the α-amylase inhibition assay, the MTT cell viability assay, and the glucose uptake assay.

4.4.2 Cell Viability Assay

4.4.2.1 MTT Assay

The MTT assay was used to assess the viability of L6 and HepG2 cell lines upon treatment with different concentrations (10, 25, 50, 75, and 100 µg/mL) of dried methanolic rice extracts (Njavara and Rakthashali). Cells were seeded at a density of 1 × 10^4 cells/well in a 96-well plate and incubated overnight to allow adherence. After 70-80% confluency, cells were treated with different concentrations of dried methanolic extracts of Njavara and Rakthsali, dissolved in DMEM, and incubated for 24 hours. After treatment, 100 µL of MTT reagent (5 mg/mL in PBS) was added to each well in the dark,and the plate was incubated for 2 hours in a 5% CO_2 incubator at 37°C. Following incubation, 100 µL detergent (50 % DMF + 20 % SDS) was added directly to the MTT reagent to dissolve the formazan crystals. After 2 hours of incubation, absorbance wasmeasured at 570 nm using a microplate reader. Cell viability was calculated as a percentage.

Cell viability was calculated using the following formula:

$$\text{Cell Viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

4.4.3 Evaluation of Antidiabetic Activity

4.4.3.1 Alpha Amylase Inhibition Assay

The experiment involved taking different concentrations of Njavara and Rakthashali methanolic extracts, ranging from 50 -250 µg/mL.100µl of 0.5 mg/mL alpha amylase solution was added to each tube, and the mixture was incubated at 25°C for 10 minutes. Next,100 µL of 1 % soluble starch (potato starch dissolved in sodium phosphate buffer at pH 6.9) was added to each tube and incubated again at 25°C for 10 minutes. Then, 0.2 mL dinitrosalicylic acid reagent was added, and the tubes were placed in a boiling water bath at 100 °C for 5 minutes. After this, 2 mL of distilled water was added to each tube, and the control was maintained.

The absorbance was measured at 540 nm using a UV–Visible spectrophotometer. Metformin was used as the reference standard. The percentage inhibition of α-amylase activity was calculated using the following formula:

$$(\%)\text{Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

4.4.4 Glucose uptake assay

4.4.4.1 Reagent and Standard Preparation

A commercial Glucose Uptake Assay Kit (Colorimetric, ab136955, Abcam) was utilized to measure glucose uptake. Prior to opening, all small vials were briefly centrifuged at low speed. Assay Buffer 3, Extraction Buffer I, and Neutralization Buffer II were equilibrated to room temperature before use. The lyophilized reagents were reconstituted as follows: Enzyme Mix III was dissolved in 220 µL of Assay Buffer 3 and maintained on ice; Glutathione Reductase was dissolved in 1.1 mL of Assay Buffer 3; the Recycling Mix was reconstituted with 220 µL of Assay Buffer 3; and DTNB was dissolved in 1 mL of Assay Buffer 3. Reconstituted components were aliquoted into working volumes and stored at -20°C to avoid repeated freeze-thaw cycles. The 2-DG6P Standard was reconstituted with 100 µL of ultra-pure water (ddH2O) to generate a 10 mM (10 nmol/µL) stock solution.

A fresh set of standards was prepared for each assay run. A 0.1 mM (100 pmol/µL) intermediate standard solution was generated by diluting 5 µL of the 10 mM stock into 495 µL of Assay Buffer 3. From this, a 0.01 mM (10 pmol/µL) working standard was prepared by diluting 50 µL of the intermediate standard into 450 µL of Assay Buffer 3. Standard curve dilutions were then established in a clear, flat-bottom 96-well plate to yield final quantities of 0, 20, 40, 60, 80, and 100 pmol of 2-DG6P per well, each adjusted to a final volume of 50 µL using Assay Buffer 3. All standard treatments were analyzed in duplicate.

4.4.4.2 Cell Seeding, Starvation, and Treatment

L6 Cells were seeded at a density of 1,500–2,000 cells per well in a sterile 96-well plate in 100 µL of appropriate culture medium, allowed to differentiate into mature adipocytes, and maintained for an additional 4 days prior to experimentation. To increase baseline glucose uptake, the cells were washed twice with phosphate-buffered saline (PBS) and starved overnight in 100 µL of serum-free medium.

On the following day, the cells were washed three times with PBS and pre-incubated with 100 µL of Krebs-Ringer-Phosphate-Hepes (KRPH) buffer (20 mM HEPES, 5 mM KH2PO4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, pH 7.4) containing 2% bovine serum albumin (BSA) for 40 minutes to starve the cells of glucose.

The experimental groups were designated and treated as follows:

- **Sample Background Control Cells:** Glucose-starved cells were washed three times with PBS and immediately subjected to cell lysis without 2-deoxyglucose (2-DG) exposure.
- **Insulin-Stimulated Cells:** Glucose-starved control cells were stimulated with 1 µM insulin, and test cells were treated with 5 different concentrations (10,25,50,75, and 100 µg/mL) of dried methanolic rice extract (Njavara and Rakthashali) in KRPH buffer containing 2% BSA (total volume 100 µL) for 20 minutes to activate glucose transporters, followed by the addition of 10 µL of 10 mM 2-DG.
- **Control (Untreated) Cells:** Non-insulin-stimulated cells were treated directly with 10 µL of 10 mM 2-DG.

Following a 20-minute incubation with 2-DG, all treated cells were washed three times with PBS to eliminate exogenous, unabsorbed 2-DG.

4.4.4.3 Endogenous NAD(P) Degradation and Cell Lysis

To release intracellular components and degrade endogenous NAD(P) while denaturing native enzymes, all experimental cell groups were lysed by pipetting up and down in 80 µL of Extraction Buffer I. The resulting cell lysates were subjected to a single freeze-thaw cycle and subsequently heated at 85°C for 40 minutes. The lysates were cooled on ice for 5 minutes and neutralised by adding 10 µL of Neutralisation Buffer II. The mixtures were briefly centrifuged at approximately 500 rpm for 1–2 minutes, and the clear supernatants were transferred to clean microcentrifuge tubes. Where necessary to fall within the standard curve dynamic range, supernatants were diluted 1:10 by mixing 5 µL of sample with 45 µL of Assay Buffer 3.

4.4.4.4 Reaction Assays and Spectrophotometric Measurement

For the enzymatic assay, 50 µL of the prepared standard dilutions, sample background controls, insulin-stimulated samples, and untreated control samples were added to their respective wells in a 96-well plate.

First, a master Reaction Mix A was prepared for NADPH generation, consisting of 8 µL Assay Buffer 3 and 2 µL Enzyme Mix III per reaction. A 10 µL aliquot of Reaction Mix A was dispensed into each well. The plate was mixed thoroughly and incubated at 37°C for 1 hour. To degrade any remaining, unused NADP, 90 µL of Extraction Buffer I was added to each well, the plate was sealed securely with aluminium sealing tape, and it was heated at 90°C for 40 minutes. The plate was then cooled on ice for 5 minutes and neutralized by adding 12 µL of Neutralisation Buffer II.

Next, a master Reaction Mix B was prepared for the recycling amplification reaction, comprising 20 µL Glutathione Reductase, 16 µL DTNB, and 2 µL Recycling Mix per well. A 38 µL volume of Reaction Mix B was added to all standard and sample wells, and the mixture was thoroughly mixed by pipetting. Absorbance was measured at 412 nm using a microplate reader in kinetic mode at 37°C protected from light. Readings were captured every 2–3 minutes until the highest standard (100 pmol/well) reached an optical density (OD) of 1.5-2.0. A final endpoint absorbance reading was subsequently recorded for all wells.

4.4.4.5 Data Analysis

Triplicate readings for all standards, controls, and experimental samples were averaged. The mean absorbance of the blank (Standard 1) was subtracted from all readings to obtain the corrected absorbance. For the experimental samples, the sample background control values (representing cells receiving neither insulin nor 2-DG) were subtracted to eliminate background signal interference. A standard curve was constructed by plotting the corrected absorbance values against the final 2-DG6P quantities (0–100 pmol). A best-fit smooth curve and trendline equation were calculated from the standard data.

The corrected sample OD values were interpolated against the standard curve equation to determine the absolute amount of 2-DG6P (Ts) in the sample wells.

The final concentration of 2-DG uptake was calculated using the following formula:

$$\textbf{2-DG uptake} = (Ts / Sv) \times D$$

Where Ts represents the amount of 2-DG6P in the sample well interpolated from the standard curve (pmol), Sv represents the initial sample volume introduced into the reaction well (µL), and D represents the sample dilution factor applied prior to the reaction setup.

4.4.5 RNA Isolation

To maintain an RNase-free environment, all glassware and plasticware required for RNA extraction were soaked in 0.01% DEPC solution overnight and autoclaved the next day to eliminate residual DEPC. DEPC-treated water was used to prepare all reagents. Surfaces and pipettes were decontaminated with RNaseZap, and gloves were changed regularly during the process.

4.4.5.1 Procedure for RNA Isolation

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen), and the extracted RNA was stored at -80° C in a freezer (Thermo Scientific, USA).Thecells were treated with Njavara and Rakthashali methanolic extracts at concentrations of 10, 25, 50, 75, and 100 µg/mL. The results demonstrated a concentration-dependent increase in glucose uptake, with the highest uptake observed at 100 µg/mL. Based on these findings, the 100 µg/mL concentration was selected as the optimum dose for RNA isolation.

Cell Collection:

- Cells were collected for RNA isolation on day 2, 1 mL TRIzol™ (Invitrogen) under aseptic conditions in a laminar air flow hood.
- Aspirate the media from the well, and 1ml of TRIzol™ reagent was added directly to the well to lyse the cells.
- Following lysis, the lysate was transferred to an Eppendorf tube and stored at -80◦C (Thermo Scientific, USA)

Extraction:

- The sample was vortexed and incubated at room temperature for 5 min. Chloroform was added at 200 µL per mL of TRIzol™, shaken vigorously by hand for 15 s, and incubated at room temperature for 3 min.
- Centrifugation was performed at 12,000 × g for 15 min at 4°C to separate the phases. The upper aqueous phase was transferred to 1.5 mL Eppendorf tubes. RNA was precipitated by mixing with 0.5 mL isopropanol per mL of TRIzol™.
- The mixture was incubated at room temperature for 10 min and centrifuged at 12,000 × g for 10 min at 4°C.
- The supernatant was removed, and the pellets were washed with 1 mL of 75% ethanol prepared in DEPC-treated water.
- The samples were vortexed once and centrifuged at 2000 rpm for 4 minutes at 4°C.The supernatant was discarded, and the pellet was air-dried for 5 min at room temperature.
- Pellets were dissolved in 50 µL DEPC-treated water, transferred into two 0.5 mL tubes at 10 µL each, and stored at -20°C.
- The concentration and purity of isolated total RNA were determined using a Biophotometer. RNA integrity was assessed through 1% agarose gel electrophoresis. Only samples exhibiting A260/A280 ratios between 1.8 and 2.0 with distinct 28S and 18S rRNA bands were selected for cDNA synthesis.

4.4.6 cDNA Synthesis

The first strand complementary DNA (cDNA) was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA). iScript cDNA Synthesis Kit provides a sensitive and

easy-to-use solution for two-step reverse transcription quantitative PCR (RT-qPCR). This kit includes three tubes containing all the reagents required for successful reverse transcription. The individual components of the cDNA Synthesis Kit and their respective volumes are given in Table 1.

Table 1: Components of the cDNA synthesis reaction mixture

Components	Volume per reaction
5xiScript Reaction Mix	4µL
iScriptReverse Transcriptase	1µL
RNA template	5µL
Nuclease- free water	10µL
Total Volume	20µL

The reaction was incubated in a thermal cycler with the following conditions:-25 °C for 5 minutes (primer annealing) 46 °C for 20 minutes (reverse transcription), 95 °C for 1 minute (inactivation of reverse transcriptase). The synthesized cDNA was stored at -20 °C and diluted 10 times before use in qPCR reactions.

4.4.7 Primers Used for the Study

The primer sequences used for quantitative real-time PCR (qRT-PCR) analysis were as follows: for the GLUT 4 gene, the forward primer sequence was 5′-GGT CTC CTC TGA CTT CAA CA-3′ and the reverse primer sequence was 5′-AGC CAA ATT CGT TGT CAT AC-3′. GAPDH was used as the reference (housekeeping) gene, with the forward primer sequence 5′-CCATCCTGATGACTGTGGCTCT-3′ and the reverse primer sequence 5′-GCCACGATGAACCAAGGAATGG-3′. The annealing temperature of the primers was optimized by gradient PCR over a temperature range of 56–60°C.

4.4.8 Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time PCR (qPCR) was performed for gene expression analysis using SYBR Green-based chemistry on a Bio-Rad CFX96 system (Bio-Rad Laboratories,USA). The 20 µL reaction mixture included 2 µL of 10x diluted cDNA, 10 µL of 2X SYBRGreen Master Mix, (2x). Quantitative Real-Time PCR (qPCR) was performed for gene 5 µL each of forward and reverse

primers (10 picomoles), and nuclease-free water to volume. The reaction was incubated in a thermal cycler with thefollowing conditions:-

- 95 °C for 3 minutes (initial denaturation)
- 40 cycles of:- 95 °C for 10 seconds (denaturation), 60 °C for 30 seconds (annealing), 72 °C for 30 seconds (extension). At the end of each run, melt curve analysis was performed to confirm the specificity of the amplified products.

4.4.8.1 Data Analysis

The relative expression levels of the target genes were determined using the 2^(-ΔΔCt) method. The housekeeping gene was used as an internal reference for normalisation. All reactions were performed in technical triplicate, and the mean Ct values were used for subsequent analysis.Fold changes in gene expression were calculated by comparing the Ct values of the samples with the control sample.

Calculations: Δ Ct = Ct (Target Gene) - Ct (Reference Gene), ΔΔ CT = Δ Ct (Test) - ΔCt (Control), Fold Change = 2^(-ΔΔCt)

4.4.8.2 Statistical Analysis

All RealTime-PCR reactions were performed in triplicates. Mean Ct values were used for the calculation of ΔCt, ΔΔCt, and fold-change values. Results were expressed as mean ± standard error (SE). Statistical analyses and graphical representations were performed using Microsoft Excel.

Chapter 5
Results

5.1 Preparation of Methanolic Rice Extracts

The methanolic extracts of the traditional medicinal rice varieties Njavara and Rakthashali were prepared for subsequent in vitro antidiabetic evaluation. Finely powdered rice samples were extracted using methanol, as illustrated in Figure 2. The resulting extracts were concentrated and lyophilized to obtain a dry powder, which was stored until further use. Prior to each experiment, the required quantity of the lyophilized extract was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions at the desired concentrations for biological assays.



Figure 2: Methanolic extract of Njavara and Rakthashali

The prepared methanolic extracts were subsequently used for the α-amylase inhibition assay, MTT cytotoxicity assay, glucose uptake assay, and GLUT4 gene expression analysis

5.2 Alpha Amylase Inhibition Assay

The α-amylase inhibitory activity of the methanolic extracts of Rakthashali and Njavara rice was evaluated at concentrations ranging from 50–250 µg/mL using the DNS spectrophotometric method, with Metformin as the standard. The results are presented in Table 2 and Figure 3.

Table 2: Percentage α-amylase inhibition of methanolic extracts of Rakthashali and Njavara rice in comparison with the reference standard (Metformin)

Concentration (µg/ml)	Rakthashali (%)	Metformin (%)	Njavara (%)
50	25.3	24.6	30.1
100	41.2	39.7	48.9
150	52.8	49.5	60.4
200	62.1	58.4	69.8
250	69.5	66.1	76.4

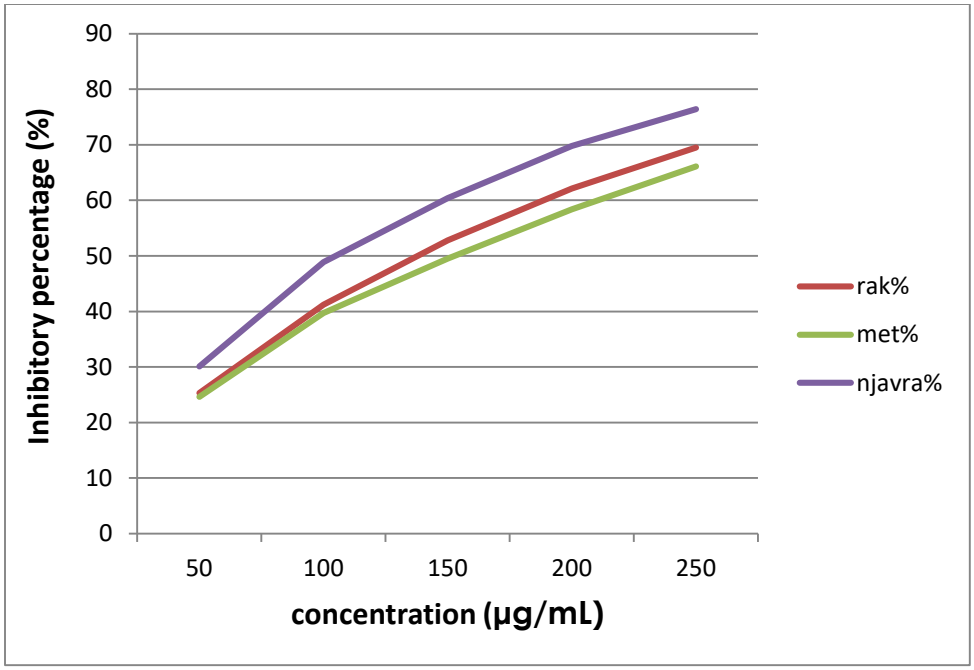


Figure 3: α-amylase inhibitory activity of Rakthashali and Njavara methanolic extracts

The methanolic extracts exhibited a concentration-dependent increase in α-amylase inhibitory activity. The methanolic extract of Njavara showed the highest inhibitory activity, with inhibition increasing from 30.1% to 76.4%, while the methanolic extract of Rakthashali showed inhibition ranging from 25.3% to 69.5%. Metformin exhibited inhibitory activity ranging from 24.6% to 66.1% over the same concentration range. At all tested concentrations, the Njavara methanolic extract exhibited higher α-amylase inhibitory activity than the Rakthashali methanolic extract and the standard.

5.3 Cell Viability Assay

After measuring the optical density of each well, the cell viability of each plant extract is calculated. The percentage of cell viability was calculated using the following formula:

$$Cell\ viability\ (\%) = \frac{Mean\ OD\ of\ test - Mean\ OD\ of\ blank}{Mean\ OD\ of\ Vehicle\ control - Mean\ OD\ of\ blank} \times 100$$

5.3.1 Assessment of Cytotoxic Effects of Rakthashali Methanolic Extract on HepG2 Cell Line

The cytotoxic potential of Rakthashali methanolic extract against HepG2 cells was evaluated using the MTT assay. The assay measures the metabolic activity of viable cells, which is directly proportional to the number of living cells. HepG2 cells were treated with different concentrations (10, 25, 50, 75, and 100 µg/ml) of Rakthashali methanolic extract, as shown in Table 3.

Table 3: Percentage cell viability of HepG2 cells treated with Rakthashali methanolic extract

Group	% Viability	SD
Control	100	0.009
Positive control (Doxorubicin 3.5µg/ml)	10.25	0.006
10 µg/ml	93.73	0.008
25 µg/ml	88.62	0.011
50 µg/ml	69.89	0.007
75 µg/ml	55.81	0.012
100 µg/ml	48.61	0.01

The results demonstrated a concentration-dependent reduction in cell viability following treatment with Rakthashali methanolic extract. The untreated control showed 100% cell viability, while the positive control exhibited 10.25% viability. At concentrations of 10, 25, 50, 75 and 100 µg/ml, the percentage cell viability was 93.73%, 88.62%, 69.89%, 55.81% and 48.61%, respectively. These findings indicate that increasing concentrations of the Rakthashali methanolic extract progressively reduced HepG2 cell viability.

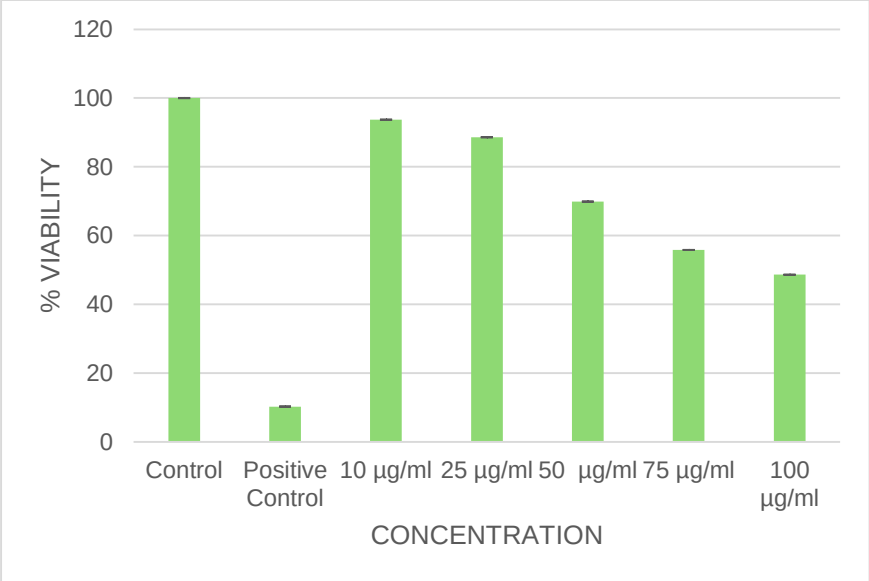


Figure 4: Dose-dependent effect of Rakthashali methanolic extract on HepG2 cell viability

Figure 4 shows that the graphical representation confirms the trend observed in Table 3, with a gradual reduction in cell viability as the extract concentration increased.

Table 4: Cell viability of Rakthashali methanolic extract at different concentration in HepG2 cells

Concentration	Cell Viability (%)
10µg/ml	93.73
25µg/ml	88.62
50µg/ml	69.89
75µg/ml	55.81
95.2µg/ml	50
100µg/ml	48.61

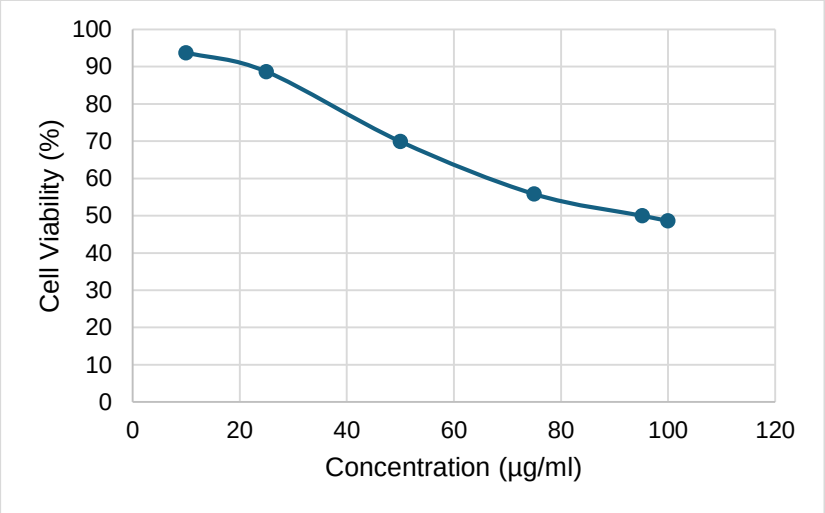


Figure 5: Dose-responsive curve showing the IC-50 of Rakthashali methanolic extract in HepG2 cells

The IC_{50} value was determined from the dose-response curve generated by plotting extract concentration against percentage cell viability, from Table 4 and Figure 5. The IC_{50} value of Rakthashali methanolic extract against HepG2 cells was calculated to be 95.2 µg/ml, indicating that this concentration reduced cell viability by approximately 50%. Doxorubicin was used as the standard drug for evaluating cytotoxicity activity. The reported IC_{50} range of doxorubicin was 0.5-12.5 µM, and 6 µM (3.5 µg/ml) was selected.

5.3.2 Assessment of Cytotoxic Effects of Rakthashali Methanolic Extract on L6 Cell Line

The cytotoxic effect of Rakthashali methanolic extract on L6 skeletal muscle cells was evaluated using the MTT assay.

Table 5. Percentage Cell Viability of L6 Cells Treated with Rakthashali Methanolic Extract

Concentration	% viability	SD
Control	100	0.09
Positive control (Doxorubicin 8.7µg/ml)	10.25	0.06
10 µg/ml	70.22	0.08
25 µg/ml	62.05	0.07
50 µg/ml	58.38	0.06
75 µg/ml	52.98	0.09
100 µg/ml	47.77	0.08

The assay measures the metabolic activity of viable cells, and the reduction of MTT to formazan crystals reflects the number of living cells. L6 cells were treated with different concentrations (10, 25, 50, 75 and 100 µg/ml) of Rakthashali methanolic extract as presented in Table 5, and the percentage cell viability was determined after incubation. The obtained percentage cell viability values are presented in Table 5.

The results demonstrated a concentration-dependent reduction in L6 cell viability following treatment with the methanolic extract. The untreated control exhibited 100% cell viability, whereas the positive control showed 10.25%. The percentage cell viability at 10, 25, 50, 75 and 100 µg/ml was 70.22 ± 0.08%, 62.05 ± 0.07%, 58.38 ± 0.06%, 52.98 ± 0.09% and 47.77 ± 0.08%, respectively. These findings indicate that increasing concentrations of Rakthashali methanolic extract progressively reduced L6 cell viability.

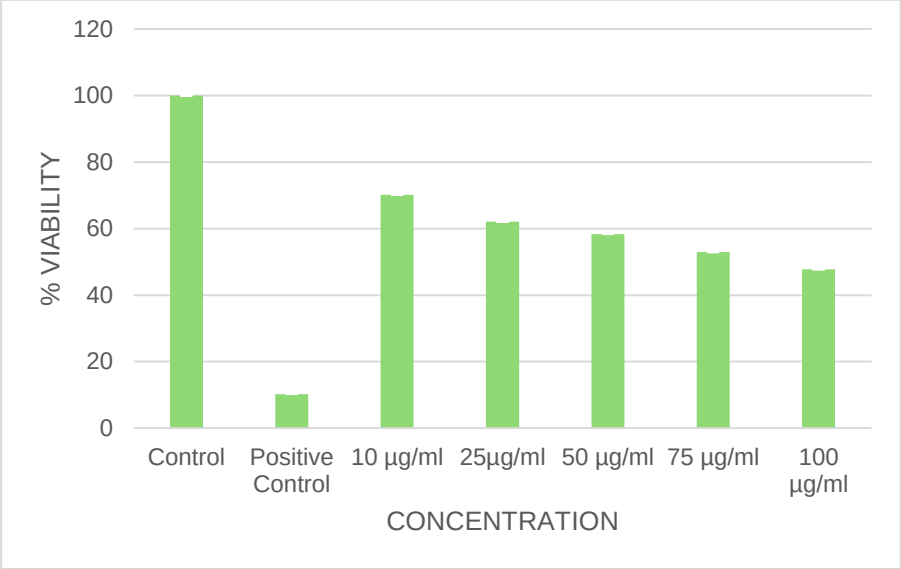


Figure 6: Effect of Rakthashali Methanolic Extract on L6 Cell Viability

Figure 6 illustrates the concentration-dependent decrease in L6 cell viability following treatment with Rakthashali methanolic extract. The graph demonstrates a gradual reduction in cell viability with increasing extract concentration, confirming the cytotoxic effect observed in the MTT assay.

Table 6: Cell viability of Rakthashali methanolic extract at different concentrations in L6 cells

Concentration	Cell Viability (%)
10µg/ml	70.22
25µg/ml	62.05
50µg/ml	58.38
75µg/ml	52.98
89.3µg/ml	50
100µg/ml	47.77

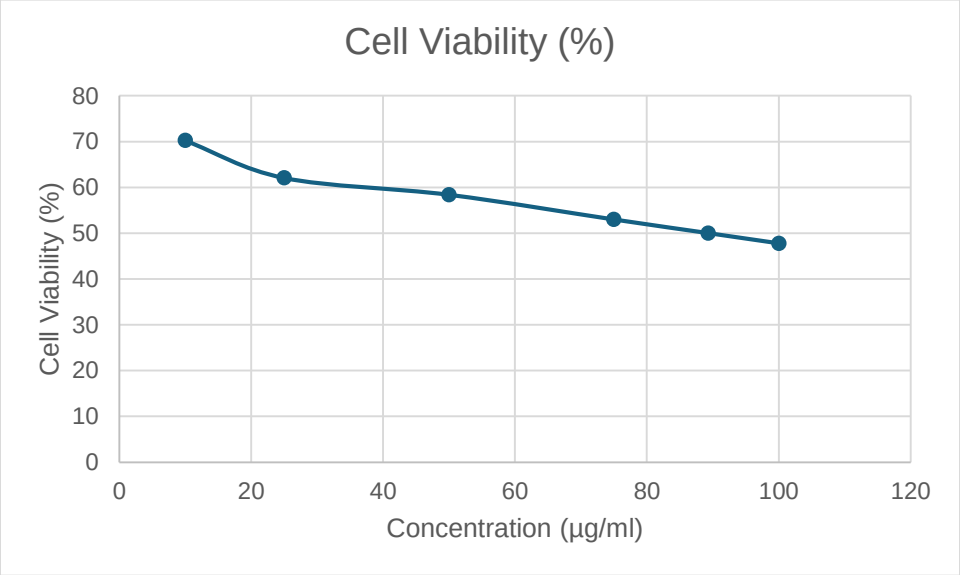


Figure 7: Dose–Response Curve Showing the IC_{50} of Rakthashali Methanolic Extract on L6 Cells

The dose-response curve, as illustrated in Figure 7, was used to determine the IC_{50} value of Rakthashali methanolic extract against L6 cells (Table 6). The calculated IC_{50} value was 89.3 µg/ml, indicating that this concentration reduced L6 cell viability by approximately 50%. The results demonstrate that Rakthashali methanolic extract exhibits concentration-dependent cytotoxicity toward L6 cells.

5.3.3 Assessment of Cytotoxic Effects of Njavara Methanolic Extract on HepG2 Cell Line

The cytotoxic potential of Njavara methanolic extract was investigated against HepG2 cells using the MTT assay. Cell viability was assessed after treatment with different concentrations (10, 25,

50, 75 and 100 µg/ml) of the extract. The percentage cell viability values obtained from the assay are summarized in Table 7.

Table 7: Percentage Cell Viability of HepG2 Cells Treated with Njavara Methanolic Extract

Group	% Viability	SD
Control	100	0.009
Positive control (Doxorubicin 3.5 µg/ml)	10.25	0.006
10µg/ml	86.12	0.008
25µg/ml	73.16	0.011
50µg/ml	66.18	0.007
75µg/ml	56.73	0.012
100µg/ml	45.61	0.01

The results showed that Njavara methanolic extract caused a progressive decline in HepG2 cell viability as the concentration increased. The untreated control maintained 100% cell viability, while the positive control exhibited 10.25% viability. Cell viability decreased from 86.12 ± 0.008% at 10 µg/ml to 45.61 ± 0.010% at 100 µg/ml. This gradual reduction in viable cells indicates that the extract exerted a concentration-dependent inhibitory effect on HepG2 cell growth.

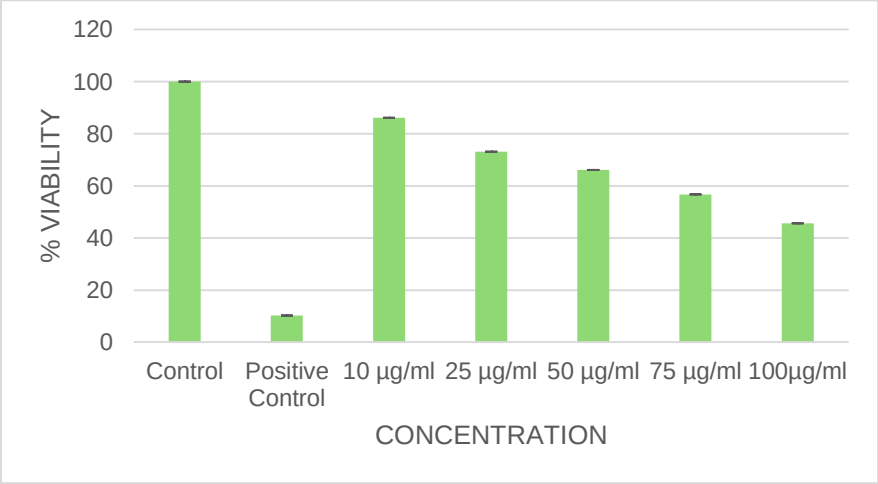


Figure 8: Dose-dependent effect of Njavara methanolic extract on HepG2 cell viability

The graphical representation in Figure 8 clearly shows that cell viability decreased steadily with increasing extract concentration, consistent with the observations in Table 7.

Table 8: Cell viability of Njavara methanolic extract at different concentrations in HepG2 cells

Concentration	Cell Viability (%)
10µg/ml	86.12
25µg/ml	73.16
50µg/ml	66.18
75µg/ml	56.73
90.1µg/ml	50
100µg/ml	45.61

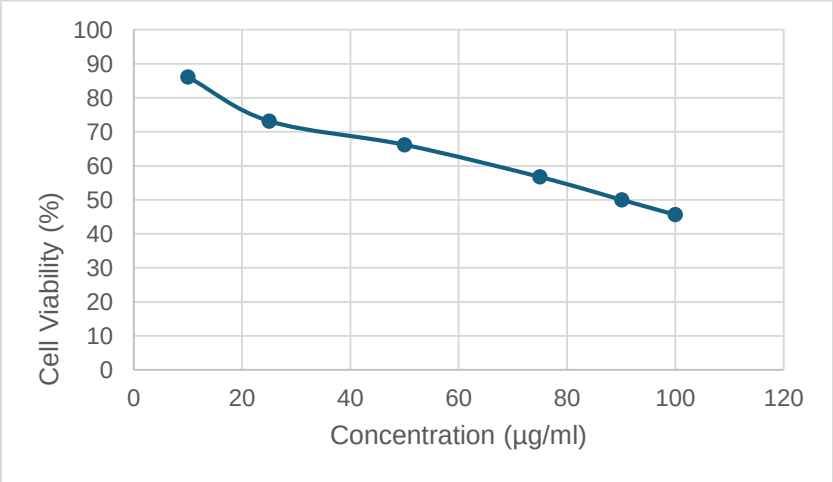


Figure 9: Dose–Response Curve Showing the IC_{50} of Njavara Methanolic Extract on HepG2 Cells

The dose-response analysis was performed to determine the IC_{50} value of Njavara methanolic extract. The calculated IC_{50} value was 90.1 µg/ml, indicating that this concentration reduced HepG2 cell viability by approximately 50%. These findings are represented in Table 8 and Figure 9, which suggest that the Njavara methanolic extract exhibits appreciable cytotoxicity against HepG2 cells.

5.3.4 Assessment of Cytotoxic Effects of Njavara Methanolic Extract on L6 Cell Line

The cytotoxic effect of Njavara methanolic extract on L6 skeletal muscle cells was determined using the MTT assay. Cells were exposed to increasing concentrations of the extract (10–100 µg/ml), and cell viability was assessed by mitochondrial metabolic activity. The percentage cell viability values are presented in Table 9.

Table 9: Percentage Cell Viability of L6 Cells Treated with Njavara Methanolic Extract

Concentration	% Viability	SD
Control	100	0.08
Positive control (Doxorubicin 8.7 µg/ml)	10.25	0.07
10µg/ml	75.38	0.09
25µg/ml	60.38	0.08
50µg/ml	65.94	0.07
75µg/ml	50.05	0.06
100µg/ml	46.33	0.09

A concentration-dependent decrease in L6 cell viability was observed following treatment with Njavara methanolic extract. Compared with the untreated control (100% viability), the treated groups exhibited progressively lower cell viability as the concentration increased. Cell viability declined from 75.38 ± 0.09% at 10 µg/ml to 46.33 ± 0.09% at 100 µg/ml, demonstrating the extract's inhibitory effect on cell survival.

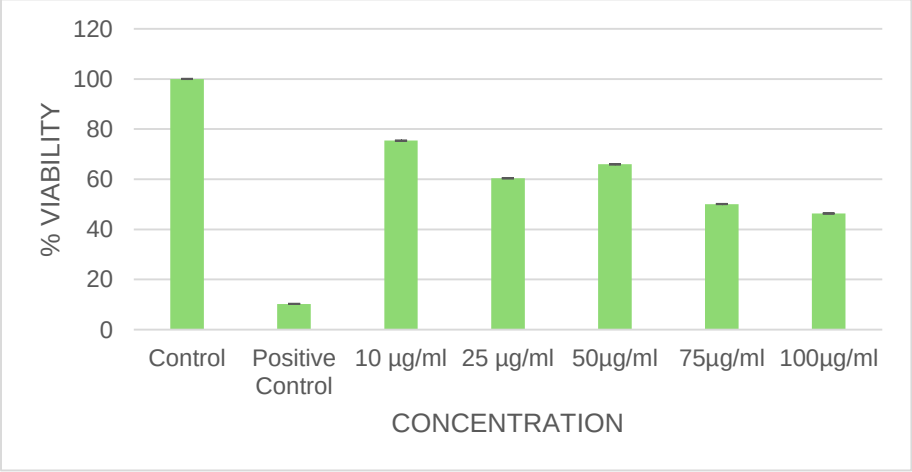


Figure 10: Effect of Njavara Methanolic Extract on L6 Cell Viability

Figure 10 illustrates the reduction in L6 cell viability following exposure to increasing concentrations of Njavara methanolic extract. The observed trend confirms that higher concentrations of the extract are associated with greater cytotoxicity in L6 cells.

Table 10: Cell viability of Njavara methanolic extract at different concentrations in L6 cells

Concentration	Cell Viability (%)
10µg/ml	75.38
25µg/ml	60.38
50µg/ml	65.94
75µg/ml	50.05
75.3µg/ml	50
100µg/ml	46.33

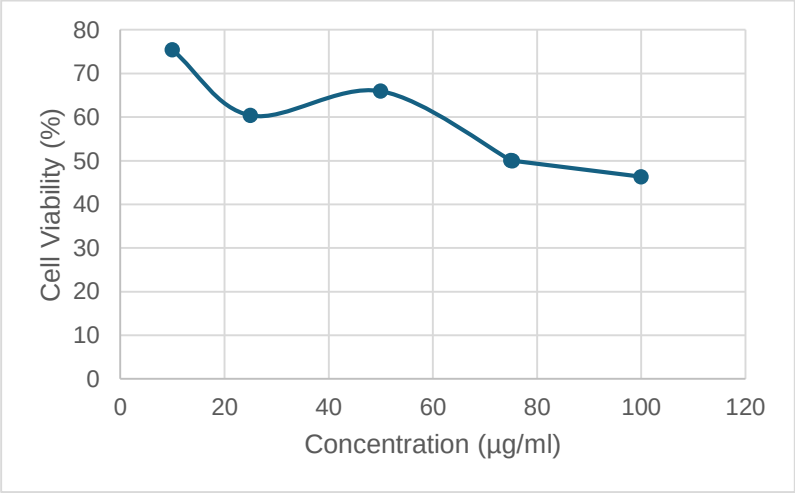


Figure 11: Dose–Response Curve Showing the IC_{50} of Njavara Methanolic Extract in L6 Cells

The IC_{50} value was estimated from the dose-response curve, Figure 11, generated using the percentage cell viability data. The calculated IC_{50} value was 75.3 µg/ml, indicating that Njavara methanolic extract reduced L6 cell viability by 50% at this concentration (Table 10). The lower IC_{50} value compared with the HepG2 assay suggests that L6 cells were more sensitive to the extract under the experimental conditions.

5.4 Glucose uptake assay

The glucose uptake assay was performed to evaluate the ability of Rakthashali and Njavara methanolic extracts to enhance cellular glucose utilization. A standard calibration curve was generated using 2-DG6P standards (Table 11) to accurately quantify glucose uptake. The assay provides a reliable and sensitive method for assessing the glucose uptake-promoting activity and potential antidiabetic efficacy of the methanolic extracts.

Table:11 OD reading of 2-DG6P standards

2-DG6P (pmol)	Mean OD
0	0.043
20	0.385
40	0.72
60	1.06
80	1.403
100	1.754

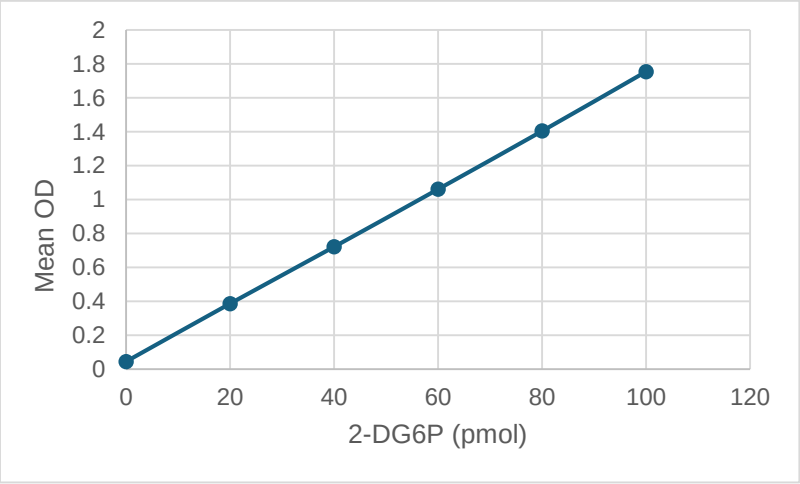


Figure 12: DG6P standard curve obtained

The standard calibration curve (Figure 12) demonstrated excellent linearity ($R^2 = 0.9999$), as calculated using the equation $R^2 = 1 - (SSres/SStot)$, where SSres is the residual sum of squares and SStot is the total sum of squares. The high R^2 value indicates that 99.99% of the variation in absorbance is explained by 2-DG6P concentration, validating the assay for accurate quantification of glucose uptake.

5.4.1 Effect of Rakthashali Methanolic Extract on Glucose Uptake in L6 Skeletal Muscle Cells

The glucose uptake activity of the methanolic extract of Rakthashali was evaluated in L6 skeletal muscle cells at different concentrations (10–100 µg/mL). As shown in Figure 13, the extract exhibited a concentration-dependent increase in glucose uptake. The uptake increased progressively from 0.46 µM at 10 µg/mL to 1.62 µM at 100 µg/mL, indicating that higher concentrations of the extract enhanced cellular glucose uptake. The maximum glucose uptake was observed at 100 µg/mL, suggesting that this concentration was the most effective in promoting glucose utilization by L6 skeletal muscle cells.

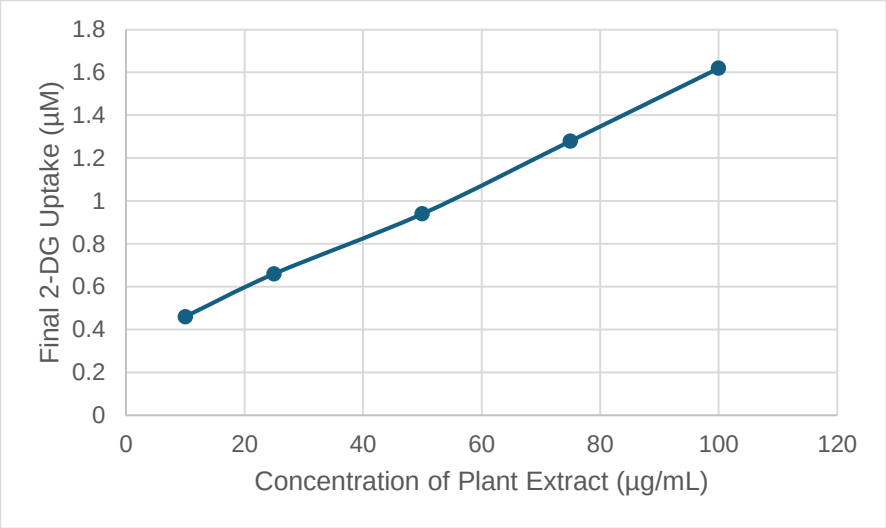


Figure 13: Concentration-dependent effect of Rakthashali methanolic extract on glucose uptake in L6 skeletal muscle cells

5.4.2 Effect of Njavara Methanolic Extract on Glucose Uptake in L6 Skeletal Muscle Cells

The glucose uptake activity of the methanolic extract of Njavara was evaluated in L6 skeletal muscle cells at concentrations ranging from 10 to 100 µg/mL. As shown in Figure 14, the extract produced a concentration-dependent increase in glucose uptake. The final 2-deoxyglucose (2-DG) uptake increased progressively from approximately 0.50 µM at 10 µg/mL to 0.80 µM, 1.25 µM, 1.60 µM, and 1.95 µM at 25, 50, 75, and 100 µg/mL, respectively. The highest glucose uptake was observed at 100 µg/mL, indicating that the Njavara methanolic extract effectively enhanced glucose uptake in L6 skeletal muscle cells in a dose-dependent manner.

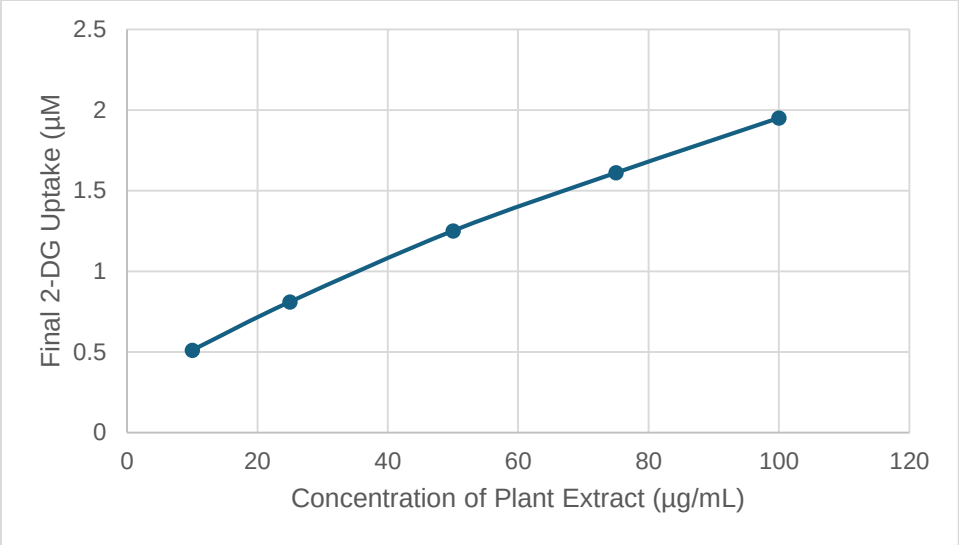


Figure 14: Concentration-dependent effect of Njavara methanolic extract on glucose uptake in L6 skeletal muscle cells

5.5 Quantitative Real-Time PCR to assess the Relative Quantification

5.5.1 Total RNA isolation and cDNA preparation

Agarose gel electrophoresis of total RNA isolated from L6 cells treated with methanolic extracts of Rakthashali and Njavara and untreated control cells revealed two distinct ribosomal RNA bands corresponding to 28S rRNA and 18S rRNA (Figure 15). Both the treated rice samples and control samples exhibited sharp, well-defined bands without noticeable smearing, indicating that the isolated RNA was intact and of high quality.

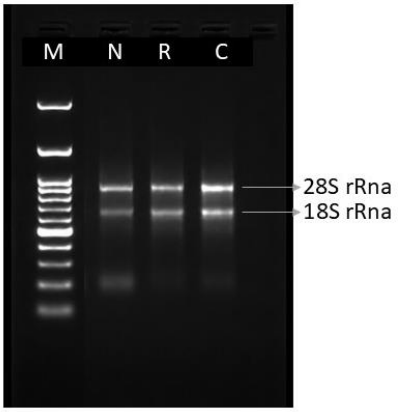


Figure 15: Agarose gel electrophoresis of total RNA isolated; M: 100bp ladder; N: L6 cells treated with Njavara methanolic extracts R: L6 cells treated with Rakthashali methanolic extracts and C: untreated control cells revealed two distinct ribosomal RNA bands corresponding to 28S rRNA and 18S rRNA

These results confirmed that the RNA samples were suitable for subsequent cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis.Complementary DNA (cDNA) was successfully synthesized from total RNA isolated from all the three samples. The quality of the synthesized cDNA and the efficiency of the primers were evaluated by quantitative real-time PCR (qRT-PCR) using GAPDH as the reference gene. The amplification profile showed a characteristic sigmoidal curve with a Ct value of 19.09, indicating successful cDNA synthesis and sufficient template for downstream gene expression analysis.

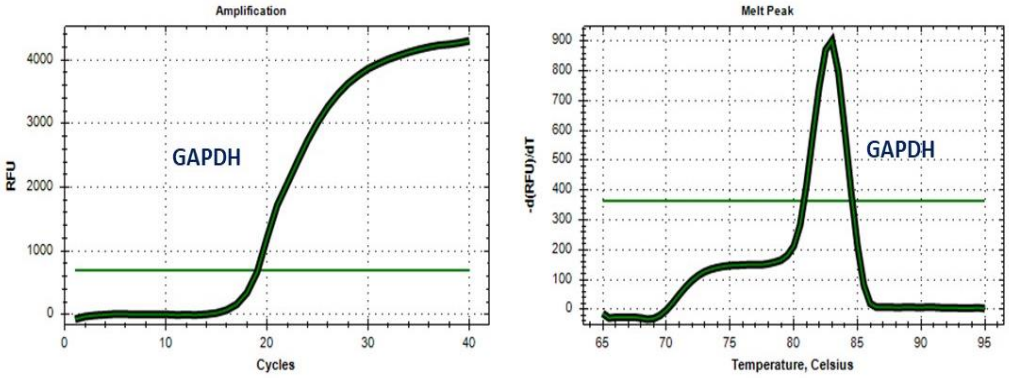


Figure 16: Representative qRT-PCR amplification and melt curve analysis of the GAPDH reference gene used for cDNA quality assessment

Melt curve analysis revealed a single, sharp peak for GAPDH amplification in both samples(Figure 16), confirming specific amplification, efficient primer performance, and the suitability of the synthesized cDNA for subsequent gene expression analysis.

5.5.2 Relative Expression of the GLUT4 Gene in L6 Cells Treated with Methanolic Extracts of Njavara and Rakthashali Rice

Based on the glucose uptake assay, 100 µg/mL was identified as the optimum concentration, as it produced the highest glucose uptake in L6 skeletal muscle cells. Therefore, L6 cells were treated with 100 µg/mL of the methanolic extracts of Njavara and Rakthashali rice for 24 hours, and the relative expression of the GLUT4 gene was quantified by qRT-PCR using the 2^−ΔΔCt method, with GAPDH serving as the internal reference gene.

As shown in the graph, the untreated control cells exhibited a normalized GLUT4 gene expression level of 1.0-fold. In contrast, treatment with the methanolic extract of Rakthashali significantly increased the relative expression of GLUT4 to approximately 1.56-fold, representing a 56% increase compared with the untreated control. (Figure 17) Furthermore, treatment with the methanolic extract of Njavara produced the highest level of GLUT4 expression, reaching approximately 1.90-fold, corresponding to a 90% increase over the control. The error bars indicate minimal variation among the experimental replicates, demonstrating good reproducibility and consistency of the qRT-PCR analysis.

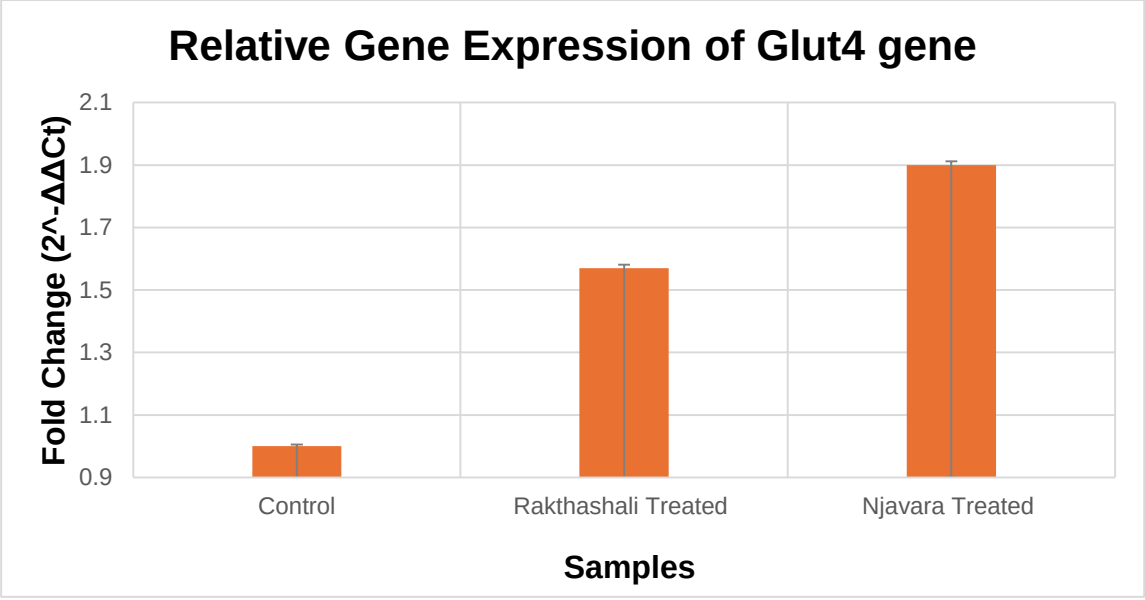


Figure 17: Relative expression of the *GLUT4* gene in L6 skeletal muscle cells treated with methanolic extracts of Njavara and Rakthashali rice

The observed increase in GLUT4 expression indicates that both rice extracts positively regulate the transcription of the glucose transporter gene in L6 skeletal muscle cells. Among the two

treatments, Njavara exhibited a greater stimulatory effect than Rakthashali, suggesting that it possesses a stronger ability to enhance glucose transporter expression. Since GLUT4 is the principal insulin-responsive glucose transporter responsible for facilitating glucose uptake into skeletal muscle cells, its upregulation is expected to improve cellular glucose utilization and maintain glucose homeostasis. These findings are consistent with the glucose uptake assay, where both extracts enhanced glucose uptake, and further suggest that the increased glucose utilization may be mediated, at least in part, through the upregulation of GLUT4 gene expression. Overall, the results demonstrate that the methanolic extracts of Njavara and Rakthashali rice possess promising antidiabetic potential by enhancing GLUT4 expression in skeletal muscle cells, with Njavara exhibiting the strongest effect.

Chapter 6

Discussion

The present study investigated the in vitro antidiabetic potential of methanolic extracts of Njavara and Rakthashali rice varieties using α-amylase inhibition, MTT cell viability, glucose uptake, and GLUT4 gene expression assays. The results demonstrated that both rice extracts possess significant antidiabetic activity in a concentration-dependent manner. Among the two, Njavara consistently exhibited superior biological activity, showing stronger α-amylase inhibition, higher glucose uptake, and greater upregulation of GLUT4 gene expression than Rakthashali. These findings support the traditional use of medicinal rice varieties as functional foods with potential therapeutic value in the management of diabetes mellitus.

The α-amylase inhibition assay demonstrated that both methanolic extracts effectively inhibited α-amylase activity, with inhibition increasing as the extract concentration increased. Njavara exhibited inhibition ranging from 30.1% to 76.4%, whereas Rakthashali showed inhibition ranging from 25.3% to 69.5%. The stronger inhibitory activity observed in Njavara suggests a greater ability to delay the enzymatic breakdown of dietary starch into glucose, thereby reducing postprandial blood glucose levels. Since inhibition of α-amylase is a well-established strategy for controlling postprandial hyperglycaemia by slowing carbohydrate digestion and glucose absorption, the higher inhibitory activity of Njavara indicates better antihyperglycemic potential. This enhanced activity may be attributed to its higher levels of phenolic compounds, flavonoids, and other antioxidant phytochemicals. Similar findings were reported by Rao et al. (2010), who demonstrated that methanolic extracts of Njavara rice bran are rich in phenolic compounds and flavonoids, contributing to strong antioxidant and biological activities. Likewise, Deepa et al. (2008) described Njavara as a unique medicinal rice variety containing valuable nutrients and bioactive constituents responsible for its therapeutic properties. In addition, Haldipur et al. (2021) reported that phenolic-rich Indian red rice genotypes exhibit significant α-amylase inhibitory activity and antihyperglycemic effects, supporting the observations of the present study.

The MTT assay revealed that both extracts reduced cell viability in a concentration-dependent manner in both HepG2 and L6 cell lines, with higher cell viability being maintained at lower extract concentrations. The IC_{50} values for Rakthashali were 95.2 µg/ml in HepG2 cells and 89.3

µg/ml in L6 cells, whereas Njavara showed IC_{50} values of 90.1 µg/ml and 75.3 µg/ml, respectively. These findings indicate that the cytotoxic effects of both extracts increased with increasing concentration. The comparatively higher IC_{50} values observed in HepG2 cells suggest lower cytotoxicity toward hepatic cells than skeletal muscle cells under the present experimental conditions. Nevertheless, both extracts maintained satisfactory cell viability at lower concentrations, making these concentrations suitable for evaluating glucose uptake and gene expression studies. These findings agree with Mosmann (1983), who established the MTT assay as a reliable method for assessing cell viability and cytotoxicity. Similarly, Rao et al. (2010) reported that methanolic extracts of Njavara rice bran exhibit dose-dependent antiproliferative activity associated with their high phenolic and flavonoid content. These observations indicate that although the extracts possess significant biological activity, selection of an appropriate concentration is essential to maximize biological efficacy while minimizing cytotoxic effects.

The glucose uptake assay further demonstrated that both methanolic extracts significantly enhanced glucose uptake in L6 skeletal muscle cells in a concentration-dependent manner. Njavara increased glucose uptake from 0.50µM to 1.95 µM, whereas Rakthashali increased glucose uptake from 0.46 µM to 1.62 µM across the tested concentration range. Although the difference between the two varieties was relatively small, Njavara consistently exhibited greater glucose uptake than Rakthashali, suggesting stronger insulin-mimetic or insulin-sensitizing activity. Skeletal muscle is the principal site of insulin-mediated glucose disposal and therefore plays a critical role in maintaining glucose homeostasis. Consequently, enhanced glucose uptake in L6 cells reflects improved peripheral glucose utilization, an important mechanism for the control of diabetes mellitus. DeFronzo et al. (1981) reported that skeletal muscle accounts for the majority of insulin-stimulated glucose uptake in the body. Furthermore, Abdul-Ghani and DeFronzo (2010) emphasized that impaired glucose uptake in skeletal muscle is one of the major factors contributing to insulin resistance and type 2 diabetes. Thus, the enhanced glucose uptake observed in the present study further supports the potential of these rice extracts in improving glucose metabolism.

During adipocyte maturation, the transcriptional activity of key insulin-signalling genes, including *Peroxisome proliferator-activated receptor gamma* (*Pparγ*) and *Glucose transporter-4*(*Glut-4*), is upregulated to promote glucose and fatty acid uptake, processes that are essential for adipogenesis (Kim et al., 2012). GLUT-4 is the principal insulin-responsive glucose transporter in adipose tissue

and skeletal muscle. Upon insulin stimulation, GLUT-4-containing intracellular vesicles translocate to the plasma membrane, thereby facilitating cellular glucose uptake (Reaven et al., 1988). In type 2 diabetes mellitus (T2DM), insulin resistance disrupts this process by reducing GLUT-4 expression and impairing its translocation to the plasma membrane, resulting in diminished glucose uptake in insulin-sensitive tissues (Xu et al., 2017). Consequently, reduced GLUT-4 expression and impaired insulin responsiveness are considered hallmark features of T2DM. Previous studies using differentiated human adipocytes and mature 3T3-L1 adipocytes have similarly reported decreased GLUT-4 mRNA and protein expression, accompanied by defective GLUT-4 translocation under insulin-resistant conditions (Derici et al., 2021).

To investigate the molecular mechanism underlying the enhanced glucose uptake, the relative expression of the GLUT4 gene was analysed in L6 skeletal muscle cells treated with 100 µg/mL of the methanolic extracts for 24 h. This concentration was selected based on the glucose uptake assay, as it produced the highest glucose uptake without affecting cell viability. Quantitative real-time PCR analysis revealed that both rice extracts upregulated GLUT4 gene expression compared with the untreated control. Rakthashali treatment resulted in approximately 1.56-fold upregulation of GLUT4, whereas Njavara produced the highest expression of approximately 1.90-fold relative to the control. The greater induction of GLUT4 expression by Njavara closely corresponds with its superior glucose uptake activity, indicating that the enhanced glucose utilization observed in L6 cells is likely mediated through increased expression of this insulin-responsive glucose transporter. Since GLUT4 is responsible for insulin-dependent glucose transport into skeletal muscle cells, increased expression of this transporter facilitates greater cellular glucose uptake and contributes to improved glucose homeostasis. Therefore, the molecular findings corroborate the results of the glucose uptake assay and provide mechanistic evidence that the antidiabetic activity of both rice extracts, particularly Njavara, may be mediated through the upregulation of GLUT4 expression.

The consistently higher biological activity observed for Njavara throughout this study may be attributed to its phytochemical composition. Medicinal rice varieties are known to contain high levels of phenolic acids, flavonoids, γ-oryzanol, tocopherols, and tocotrienols, all of which possess antioxidant and antidiabetic properties. These bioactive compounds help reduce oxidative stress, inhibit carbohydrate-hydrolysing enzymes, improve insulin sensitivity, and enhance glucose

transport, thereby contributing to better glycaemic control. The greater GLUT4 expression observed in Njavara-treated cells may therefore be associated with the synergistic action of these phytochemicals in activating insulin-responsive signalling pathways involved in glucose transport. Goufo and Trindade (2014) reviewed the nutritional and functional properties of coloured rice and concluded that pigmented rice varieties are excellent sources of bioactive compounds with potential health benefits, including antidiabetic activity. Likewise, Min et al. (2012) reported that pigmented rice bran contains high concentrations of antioxidant phytochemicals, including proanthocyanidins and anthocyanins, which may improve metabolic health and reduce oxidative stress.

Altogether, the findings of the present study demonstrate that both Njavara and Rakthashali methanolic extracts possess promising in vitro antidiabetic activity, as evidenced by effective α-amylase inhibition, enhanced glucose uptake, and upregulation of GLUT4 gene expression in L6 skeletal muscle cells. Among the two rice varieties, Njavara consistently exhibited superior biological activity, including stronger α-amylase inhibitory activity, greater glucose uptake, and higher GLUT4 expression, indicating comparatively greater antidiabetic potential. Although both extracts exhibited concentration-dependent cytotoxicity at higher concentrations, lower concentrations maintained good cell viability while retaining significant biological activity. Collectively, these findings suggest that traditional medicinal rice varieties, particularly Njavara, represent valuable natural sources of antidiabetic phytochemicals capable of improving glucose metabolism through multiple complementary mechanisms, including inhibition of carbohydrate digestion and enhancement of skeletal muscle glucose transport. However, further studies involving the isolation and characterization of the active phytochemicals, elucidation of the signalling pathways regulating GLUT4 expression, and in vivo validation are necessary to confirm these findings and establish their therapeutic potential.

Summary and Conclusion

The present study evaluated the in vitro antidiabetic potential of methanolic extracts of the medicinal rice varieties Njavara and Rakthashali using α-amylase inhibition, MTT cell viability, glucose uptake, and GLUT4 gene expression assays. Both rice extracts exhibited significant concentration-dependent α-amylase inhibitory activity, indicating their ability to delay carbohydrate digestion and reduce postprandial glucose release. Among the two varieties, Njavara consistently demonstrated superior biological activity, exhibiting greater α-amylase inhibition (76.4%) than Rakthashali (69.5%). The MTT assay revealed concentration-dependent cytotoxicity in both HepG2 and L6 cell lines; however, lower concentrations maintained satisfactory cell viability, confirming their suitability for evaluating glucose uptake and gene expression.

Both extracts significantly enhanced glucose uptake in L6 skeletal muscle cells, with Njavara (1.95 μM) showing slightly greater glucose uptake than Rakthashali (1.62 μM). To investigate the underlying molecular mechanism, the relative expression of the GLUT4 gene was quantified by qRT-PCR. Treatment of L6 cells with 100 μg/mL of the methanolic extracts for 24 h significantly upregulated GLUT4 expression, with Rakthashali producing approximately 1.56-fold and Njavara producing approximately 1.90-fold higher expression than the untreated control. The enhanced GLUT4 expression closely correlated with the increased glucose uptake observed in L6 cells, suggesting that both extracts improve glucose utilization by promoting the expression of the insulin-responsive glucose transporter.

Overall, the findings demonstrate that the methanolic extracts of Njavara and Rakthashali possess promising in vitro antidiabetic activity through multiple complementary mechanisms, including inhibition of carbohydrate digestion, enhancement of skeletal muscle glucose uptake, and upregulation of GLUT4 gene expression. Among the two varieties, Njavara consistently exhibited superior antidiabetic efficacy, indicating its greater therapeutic potential for improving glucose homeostasis. Although both extracts exhibited concentration-dependent cytotoxicity at higher doses, the optimum concentration of 100 μg/mL maintained good cell viability while producing maximum glucose uptake and GLUT4 induction. These findings provide scientific evidence supporting the traditional medicinal use of Njavara, particularly as a functional food with potential applications in diabetes management.

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