Evaluation of the Antidiabetic Activity of Northeast Indian variety of *Zingiber rubens* and Exploring the Mechanism of Action

Thesis Submitted By

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CERTIFICATE

*This is to certify that the thesis entitled "*Evaluation of the Antidiabetic Activity of Northeast Indian variety of *Zingiber rubens* and Exploring the Mechanism of Action*." submitted by Shri Sujit Das , who got his name registered on 28-05-2018 for the award of Ph. D. (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the joint supervisions of Prof. Pallab Kanti Haldar and Dr. Manas Bhowmik and that neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.*

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Statement of Originality

I, Sujit Das registered on 28.05.2018 to do hereby declare that this thesis entitled "Evaluation of the Antidiabetic Activity of Northeast Indian variety of *Zingiber rubens* and Exploring the Mechanism of Action." contains literature survey and original research work done by the undersigned candidates as part of Doctoral studies. All information in this thesis have been obtained and presented in accordance with existing academic rules ethical conduct. I declare that as required by thesis rules and conduct, I have fully cited and referred all materials and results that are not original to this work. I also declare that I have checked this thesis as per the Policy on Anti-plagiarism, Jadavpur University, 2019" and the level of similarity as checked by iThenticate software is 6%.

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Declaration

I hereby declare that my research work embodied in this Ph.D. (Pharmacy) thesis entitled "Evaluation of the Antidiabetic Activity of Northeast Indian variety of *Zingiber rubens* and Exploring the Mechanism of Action**."** have been carried out by me in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700032 India, under the joint supervision of Prof. Pallab Kanti Haldar, Professor, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032 & Director, School of Natural Product Studies, Jadavpur University, Kolkata-700032 and Dr. Manas Bhowmik, Associate Professor, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India. I also confirmed that this work is original and has not been submitted partly of in full for any other degree of diploma to this or other University or Institute.

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Contents

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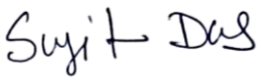

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Preface

The present thesis entitled "Evaluation of the Antidiabetic Activity of Northeast Indian variety of *Zingiber rubens* and Exploring the Mechanism of Action." comprises the work done by the author in Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata for the degree of Doctor of Philosophy in Pharmacy.

Over the past two decades, there has been an enormous growth in the use of medicinal plants in the therapeutic management of various diseases. Drug corporations and research institutions are focusing their efforts on the untapped potential of the plant kingdom in their search for novel medications and lead chemicals. The traditional healthcare system which is mostly based on botanical sources is still used by about 80% of the worldwide population.

Plant extracts have been recommended as therapies in the Indian traditional healthcare system from ancient times. All significant medical traditions including Ayurveda, Unani and homoeopathy depend primarily on medications of plant origin. The basis of the Indian traditional system is made up of low toxicity, simple availability of herbal products and frequent use of practitioners an ingrained faith particularly in rural regions and a strong public desire to employ medicinal plants for therapeutic purposes. Some medicinal food plants were selected from the Zingiberaceae family which depending on the potency of herbal research and are traditionally of use in the North-East region as a treatment for a wide range of diseases and problems.

In the present study, some medicinal food plant fruits were selected for isolation, *in-vitro*, *in-vivo*, and molecular mechanisms through modulation of the oxidative stress and glycosylated hemoglobin (HbA1c) of antidiabetic evaluation with various animal models.

Before the pharmacological evaluation, the integrated network pharmacological analysis was performed. The plant parts were also evaluated for *in-vitro* anti-diabetic activity and the efficacious fraction was used in an experimental *in-vivo* anti-diabetic animal model. Thus the thesis covered the above mentioned studies in a logical sequence with related references annexed to each chapter. In conclusion the detailed study has been linked up in a manner to justify the relation of the work to establish the antidiabetic potency of Zingiberaceae family plants especially to explore the molecular mechanism through modulation of the oxidative stress and glycosylated hemoglobin (HbA1c) and inhibition of protein kinase C.

Mr. Sujit Das

List of Abbreviation

$^1O_2$	-	Singlet oxygen
ACE	-	Angiotensin I-converting enzyme
ACN	-	Acetonitrile
AESH	-	Aqueous Ethanol Extract *Solena heterophylla*
AGI	-	Alpha Glucosidase Inhibitors
ALP	-	Alkaline Phosphatase
ALT	-	Alanine Transaminase
ANOVA	-	Analysis of Variance
AST	-	Aspartate Transaminase
ATP	-	Adenosine triphosphate
BHA	-	Butylated hydroxyanisole
BHA	-	Butylated Hydroxy Anisole
BHT	-	Butylated hydroxytoluene
CAM	-	Complementary and alternative medicine
CAT	-	Catalase
CFCP	-	Chloroform Fraction of *Cyclanthera pedata*
CFSH	-	Chloroform Fraction *Solena heterophylla*
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experiments on Animals
CuB	-	Cucurbitacin B
DNA	-	Deoxyribonucleic acid
DPP4	-	Dipeptidyl peptidase 4
DPPH	-	1,1-diphenyl-1-picrylhydrazyl
DTNB	-	5, 5′-dithio bis-2-nitro benzoic acid
EACP	-	Ethyl acetate Fraction of *Cyclanthera pedata*
EASH	-	Ethyl Acetate Fraction *Solena heterophylla*
EASH	-	Ethyl acetate of *Solena heterophylla*
EDTA	-	Ethylene diamine tetra acetic acid
EFCP	-	Ethanolic fraction of *Cyclanthera pedata*
EFSH	-	Ethanol Fraction *Solena heterophylla*
EFSH	-	Ethanolic fraction of *Solena heterophylla*
FBG	-	Fasting Blood Glucose

GAE	-	Gallic acid equivalent
GC-MS	-	Gas chromatography–mass spectrometry
GH	-	Glycoside hydrolase
GLP-1	-	Glucagon-like peptide
GLUT2	-	Glucose transporter 2
GPX	-	Glutathione peroxidase
GSH	-	Glutathione
H_2O_2	-	Hydrogen peroxide
HbA1C	-	Glycosylated hemoglobin
HFCP	-	Hexane Fraction of *Cyclanthera pedata*
HFD	-	High Fat Diet
HFSH	-	Hexane Fraction *Solena heterophylla*
HO	-	Hydroxyl
HOCl	-	Hypochlorous acid
HPLC	-	High-performance liquid chromatography
HPTLC	-	High-performance thin layer chromatography
IDDM	-	Insulin Dependent Diabetes Mellitus
IDF	-	International Diabetes Federation
LC-MS	-	Liquid chromatography-mass spectrometry
LC-MS/MS	-	Liquid chromatography-mass spectrometry-mass spectrometry
LC-QTOF-MS	-	Liquid Chromatography-Quadrupole Time-of-Flight Tandem Mass Spectrometry
MDA	-	Malondialdehyde
MGAM	-	Maltase-Glucoamylase
NADH	-	Reduced nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide
NaOH	-	Sodium Hydroxide
NBT	-	Nitrobluetetrazolium chloride
NIDDM	-	Non-Insulin Dependent Diabetes Mellitus
NIDDM	-	Non-insulin-dependent diabetes mellitus
NMR	-	Nuclear Magnetic Resonance
NO	-	Nitric Oxide
O^2	-	Superoxide
OECD	-	Organisation for Economic Co-operation and Development

OGTT	-	Oral Glucose Tolerance Test
PKC	-	Protein Kinase C
PMS	-	Phenazinemethosulphate
RNS	-	Reactive Nitrogen Species
RO	-	Alkoxyl
ROO	-	Peroxyl
ROS	-	Reactive Oxygen Species
SALP	-	Serum Alkaline Phosphatase
SEM	-	Standard Error of Mean
SGLT1	-	Sodium/glucose transporter
SGOT	-	Serum glutamic oxaloacetic transaminase
SGPT	-	Serum glutamic pyruvic transaminase
SHF	-	*Solena heterophylla* Fraction
SI	-	Sucrase-isomaltase
SOD	-	Super oxide Dismutase
STZ	-	Streptozotocin
T1DM	-	Type 1 Diabetes Mellitus
T2DM	-	Type 2 Diabetes Mellitus
TBA	-	Thiobarbituric acid
TBARS	-	Thiobarbituric Acid Reactive Substances Assay
TCA	-	Trichloroacetic Acid
UV	-	Ultraviolet
WHO	-	World Health Organization

Dedicated
to
My Family
&
My Guide

INTRODUCTION:

Diabetes is increasingly common due to urbanization and poor lifestyle choices, with a growing impact on the health and economy of developing nations. In India, the International Diabetes Federation (IDF) projected around 50.8 million diabetics in 2010, rising to 87 million by 2030 [1-2]. Recent data indicates a rising prevalence in both urban and rural areas. Current oral hypoglycaemic medications have various side effects, including heart failure and gastrointestinal issues, highlighting the need for safer therapeutics. A multi-target therapeutic agent is necessary to address the complexities of Type 2 diabetes (T2DM). Plant extracts, known for their medicinal properties, are being explored for their potential in diabetes treatment [1-3].

Medicinal plants significantly manage and treat diabetes, offering valuable alternatives and complementing conventional therapies [1]. A notable example is Metformin, a widely prescribed oral medication that effectively lowers blood glucose levels [2]. The development of Metformin was inspired by the historical use of *Galega officinalis*, a plant known for its traditional application in treating diabetes. This fascinating herb is abundant in guanidine, a compound recognized for its hypoglycemic properties [2-3]. However, due to guanidine's toxicity, it was deemed inappropriate for clinical use. Instead, researchers synthesized alkyl biguanides, namely synthalin A and synthalin B, introduced in Europe as oral anti-diabetic agents during the 1920s. Nevertheless, these agents were eventually phased out after insulin treatment became more prevalent [5-6].

Despite the discontinuation of these early biguanides, the insights gained from using guanidine and its derivatives laid the groundwork for the eventual development of Metformin [6-9]. A remarkable array of over 400 traditional plant treatments for diabetes has been documented [10]. However, only a limited subset of these has undergone rigorous scientific and medical scrutiny to evaluate their effectiveness [11]. Promising findings have emerged, confirming the hypoglycemic effects of certain herbal extracts in both human and animal models of type 2 diabetes. Recognizing the potential of these natural remedies, the World Health Organization Expert Committee on diabetes has advocated for further exploration and investigation of traditional medicinal herbs to enhance our understanding and utilization of their therapeutic benefits [12-14].

Among the various natural options, several Indian medicinal plants have garnered attention for their antidiabetic properties. One such plant is ***Zingiber rubens***, which is widely

recognized for its availability and common usage. Research has indicated that certain species within the Bauhinia genus may possess significant antidiabetic activities [15-17]. A notable study published in the journal titled "Phytochemicals screening and electrophoretic study of seed storage Proteins of Bauhinia acuminata and Cassia occidentalis" asserts that Bauhinia acuminata exhibits antidiabetic effects; however, it has yet to undergo thorough evaluation to fully understand its potential [17-21].

In addition to its purported benefits for diabetes, ***Zingiber rubens*** is known for its utility in treating a variety of other health conditions, including biliousness, bladder stones, leprosy, venereal diseases, and asthma. This broad range of applications highlights the versatility and importance of this medicinal plant in traditional healing practices [22-26].

PLANT PROFILE

***Zingiber rubens* Roxb.** **Family: Zingiberaceae**

The natural habitat of this species extends from Sikkim in northern India to eastern India and across the region of Indo-China.

Review of Literature on *Zingiber rubens*

Zingiber rubens Roxb., commonly known as Bengal ginger, is a remarkable medicinal plant that belongs to the Zingiberaceae family. Renowned for its various therapeutic properties, this plant has been integral to numerous communities' traditional medicine and cultural practices [23]. Native to the lush landscapes ranging from the mountainous regions of Sikkim to the diverse habitats of Eastern India and Indo-China, Bengal ginger thrives in seasonally dry tropical environments. This resilient perennial plant features a rhizomatous structure, meaning it grows from underground stems that spread horizontally, allowing it to flourish even in challenging conditions [24].

Particularly endemic to the scenic regions of Meghalaya and Manipur in Northeast India, Z. rubens has been embraced by local cultures for its medicinal benefits. From soothing digestive issues to invigorating overall health, this plant is cherished for its ability to heal and enhance well-being in various traditional practices. Its rich heritage and diverse applications underscore Bengal ginger's significance in local and broader contexts [25].

The plant is a striking visual spectacle, adorned with vibrant bright red bracts that stand out against its fibrous rhizomes [25-26]. The edible section of Z. rubens reveals a delightful pale yellow flesh, beautifully speckled with dark red dots that add to its unique appearance. This medicinal plant has played a vital role in traditional healing practices, offering remedies for various ailments such as diarrhea, fever, cough, and colds [27-28].

This underscores its significance in the realm of ethnomedicine, where natural solutions have been sought for generations [29]. Despite the wealth of information found in historical texts regarding its applications, there is a notable lack of scientific validation for these reported benefits. Typically, the roots, seeds, and flowers are harnessed for their therapeutic properties, reflecting the plant's versatility in traditional medicine [30-31].

Phytochemical investigations have revealed that *Z. rubens* is rich in bioactive compounds, including flavonoids, terpenoids, saponins, tannins, alkaloids, and cardiac glycosides [32]. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of its essential oils identified 24 compounds, with monoterpenes constituting about 75.3% of the oil. The principal components include (Z)-citral (30.1%), camphene (9.7%), and zingiberene (5.3%) [33].

Studies indicate that extracts from Z. rubens exhibit significant antidiarrheal and thrombolytic activities[5]. However, antibacterial effects were not consistently observed against several Gram-positive and Gram-negative bacteria, such as *Escherichia coli* and *Staphylococcus aureus* [34-35]. The variability in antimicrobial activity may be attributed to the extraction methods used, as different solvents yield varying phytochemical profiles [24].

Recent studies have highlighted the antidiabetic potential of *Z. rubens*, specifically its hydroalcoholic extracts which demonstrated antioxidant activity and a reduction in protein kinase C (PKC) phosphorylation in diabetic models, suggesting a mechanism related to oxidative stress modulation [32]. Furthermore, the essential oil extracted from the rhizome showed antibacterial activity against cariogenic bacteria, making it a candidate for dental applications [21].

This plant typically grows in winter at elevated altitudes in the sub-Himalayan region [29] and has a significant history of medicinal use. Consequently, exploring methods for cultivating it in other regions is essential. Efforts in micropropagation have established optimal conditions for cultivating *Z. rubens*, with successful regeneration rates when using Murashige and Skoog (MS) medium supplemented with specific plant growth regulators [31].

Evaluation of genetic stability through the application of Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) markers has provided compelling evidence of the consistent uniformity among regenerated clones, even when observed over prolonged culture durations. This indicates not only the reliability of the cloning process but also assures that the genetic integrity of these clones is maintained over time [32].

Sl. No.	**Study Title**	**Model Organism / *In vitro* model**	**Study Design**	**Key Findings**	**Potential Applications**
1	Phytochemical screening, antidiarrheal, thrombolytic, and antibacterial	Gram-negative bacteria (*Escherichia coli* ATCC	In vitro and in vivo	Identified as a potential source for antidiarrheal and	Herbal medicine development for gastrointestinal issues

	effects of the methanol extract of Zingiber rubens Roxb. leaves.	25922, *Pseudomonas aeruginosa* ATCC 15422), Gram-positive (*Micrococcus luteus* ATCC 4698, *Staphylococcus aureus* ATCC 2592) ; Castor oil-induced diarhhea in swiss albino mice; Human RBCs		thrombolytic agents; no antibacterial activity noted.	
2	Comparative Study on the Extraction of Phytochemicals, Antibacterial Properties, and Synergistic Effects of Zingiber rubens with Tetracycline	Microbes from humans (*Homo sapiens*) and chickens (*Gallus domesticus*)	In vitro	Chloroform extract showed significant inhibition zones for *E. coli* (12.5 mm), *P. aeruginosa* (12 mm), and *S. aureus* (12 mm); synergistic	Alternative antibacterial agents, especially for resistant strains

				effect with tetracycline.	
3	Chemical Constituents of the Essential Oils from the Roots of Zingiber rubens Roxb. and Zingiber zerumbet (L.) Smith	N/A	GC and GC-MS analysis	Identified 24 compounds, primarily monoterpenes (75.3%); major components include (Z)-citral (30.1%), camphene (9.7%), zingiberene (5.3%).	Aromatherapy, flavoring agents, and potential therapeutic uses
4	The Anti-Cariogenic Activity, Cytotoxicity, and Chemical Constituents of Zingiber rubens Roxb.	*Lactobacillus casei* TISTR390, *Streptococcus mutans* ATCC25175, Vero cells	In vitro	Ethanolic extracts inhibited bacteria; essential oils showed no cytotoxicity with CC50 > 1,000 µg/ml; effective against cariogenic bacteria.	Dental applications and oral health products

5	Micropropagation of Zingiber rubens and Assessment of Genetic Stability Using RAPD and ISSR Markers	Laboratory-cultured *Z. rubens*	Micropropagation and molecular analysis	Successful regeneration on MS medium; confirmed genetic stability over time using RAPD and ISSR techniques.	Agricultural cultivation and conservation of genetic resources

Table 2: The literature review on *Zingiber Rubens;* table based on PubMed literature [22-32].

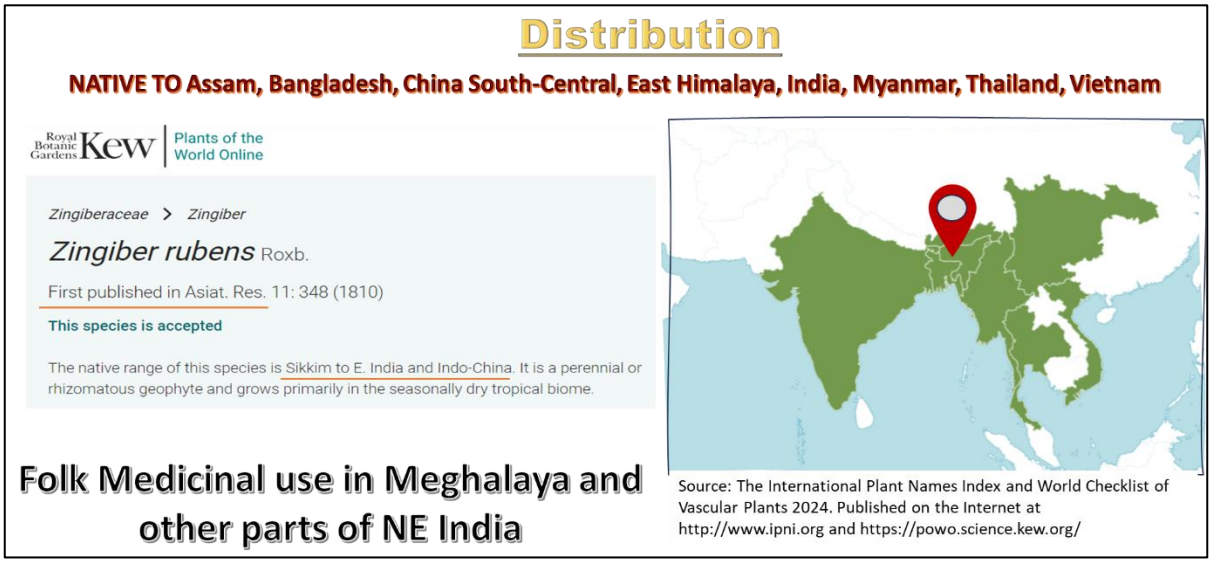

Figure 1: The Distribution of the plants and abundance in northeast India.

Despite its therapeutic promise, safety evaluations have indicated that *Z. rubens* extracts exhibit low toxicity, with CC50 values exceeding 1,000 µg/ml for most extracts, and 2.5 µg/ml for essential oils, demonstrating its potential as a safe herbal remedy [11]. The convergence of ethnobotanical uses, phytochemical diversity, and pharmacological efficacy underscores *Zingiber rubens* as a vital plant for future research and development

in medicinal applications. The antidiabetic properties of this plant have yet to be confirmed through rigorous experimental studies, and the specific molecular mechanisms underlying its potential antidiabetic effects remain unexplored. Further research is needed to investigate how this plant may contribute to blood sugar regulation and identify the biochemical pathways involved in its activity.

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1. ➢ **To validate the anti-diabetic activity of *Zingiber rubens in experimental models.***

   ✓ *In Vitro* – free radical scavenging assay.

   ✓ *In Vitro* – anti-diabetic activity.

   ✓ Integrated in silico network pharmacological analysis- Gene Disease Association Analysis.

   ✓ *In vivo*- Anti-diabetic activity

2. **To explore the underlying mechanism of action for the anti-diabetic activity of *Zingiber rubens***

   ✓ Cell culture on mouse primary cells to explore the mechanism of action.
   ✓ Inhibition Assay on Protein Kinase C phosphorylation.

PLANT MATERIALS:

An authenticated dealer procured mature rhizomes and roots of the plant from the bustling local market, ensuring high quality and adherence to standards. Following this, the plant material underwent a thorough authentication process conducted by the Botanical Survey of India, based in Gangtok, Sikkim, India 737103—this rigorous examination aimed to confirm the identity and integrity of the plant species [1-2].

To document this process, a certificate of authentication was issued (SHRC-5/02/2023-24/Tech/272), which is carefully preserved at the School of Natural Product Studies. Specifically, it can be found in the Department of Pharmaceutical Technology, located within the esteemed Jadavpur University in Kolkata, West Bengal, India [1,4]. This ensures that the authentication records are maintained in a secure and accessible manner for future reference.

Figure 1: rhizomes and roots of the plant *Zingiber rubens*

EXTRACTION OF THE PLANT MATERIALS:

The fresh rhizomes and roots were carefully harvested and thoroughly dried to preserve their essential properties. Once dried, they were meticulously ground using a mechanical grinder, transforming them into finely processed materials. This powdered crude was then skillfully packed into a thimble, which was subsequently placed into a Soxhlet apparatus designed for efficient extraction. A carefully formulated hydroalcoholic solvent mixture of 70% methanol and 30% water facilitated the extraction process. The resulting hydroalcoholic extract of Zingiber rubens (HAZR) was collected, gently dried in rotary vacuum evaporator, and then stored in an airtight container at a cool temperature of 4 °C to ensure its stability and potency for use in upcoming experiments [1,4-14]. The % yield was calculated. The dried plant crude extracts were subjected to various colour reactions to identify the presence of the phyto-constituent [12-14].

Figure 2: The soxhlation method and rotary vacuum evaporation.

Test for flavonoids [2]**:**

Ferric Chloride Test:	A small number of drops of Ferric Chloride solution were carefully introduced to the test solution, which contained the extract. Almost immediately, a vibrant green color emerged, signaling the presence of flavonoids within the sample. This

	intense coloration serves as a clear indicator of these important biochemical compounds.
Shinoda Test:	A small quantity of Ferric Chloride solution was introduced to the test solution, which contained the extract. Almost immediately, a vibrant green color emerged, signaling the presence of flavonoids. In another test, the extract was first dissolved in alcohol to create a clear solution. Next, a small piece of magnesium metal was carefully added, followed by the slow addition of concentrated hydrochloric acid, which was applied dropwise. The mixture was then gently heated, resulting in the development of a striking magenta hue, further confirming the presence of flavonoids in the extract.
Zn-HCl Acid Reduction Test:	In a small experimental setup, zinc dust was carefully introduced into the test solution, which contained the extract under investigation. This was followed by the addition of several drops of concentrated hydrochloric acid (HCl). As the two substances interacted, a few minutes passed, and the emergence of a striking red color indicated the presence of flavonoids in the solution, providing visual confirmation of their existence.
Using NaOH:	In the testing procedure, a small amount of NaOH was introduced to the solution. Upon the addition, a vibrant yellow color emerged, signaling the presence of flavonoids within the mixture.

Test for Steroids:

Salkowshi test[5]:	0.5gm of extract was dissolved in 3ml of chloroform and 3ml of concentrated sulphuric acid to form a layer. Formation of

	reddish violet color at the interface indicated the presence of steroids.

Test for Saponins[6]:

SAPONIN	A single drop of sodium hydroxide (NaOH) was carefully introduced into a 5 ml aliquot of the test solution, which contained the extract. The mixture was then shaken vigorously for a few moments, ensuring thorough mixing of the components. After shaking, the test tube was set aside for a duration of three minutes. The formation of a frothy foam resembling a honeycomb structure indicated the presence of saponins within the extract, suggesting a positive reaction.

Test for Tannins [7]:

Ferric Chloride Test:	A total of 5 ml of the extract solution was carefully measured and combined with 10 ml of water. The mixture was vigorously shaken to ensure thorough mixing. Following this, the solution was filtered to separate any undissolved particles, resulting in a clear filtrate. To this filtrate, 2 ml of Anthrone reagent was added, and the solution was observed for color changes. The appearance of a striking greenish-blue hue confirmed the presence of reducing sugars in the extract. Next, to test for tannins, 1-2 ml of the extract solution was reacted with 1 ml of a 5% ferric chloride solution. This reaction was monitored closely for the development of a distinctive greenish-black coloration, which would indicate the presence of tannins. However, despite the careful procedure, no such color change was observed, signaling the absence of tannins in the extract.

****Gelatin Test:****

In this test, 1% gelatin was incorporated into a solution containing 10% sodium chloride (NaCl). A thorough examination was conducted, and the absence of a white precipitate was noted, suggesting that tannins were not present in the solution.

****Liebermann Burchard's Test:****

For this test, two milligrams of the dried extract were measured and placed in a test tube. A few drops of acetic anhydride were carefully added to the extract. The mixture then underwent a specific heating and cooling process. Following this, one milliliter of concentrated hydrochloric acid (HCl) was introduced. The lack of a green coloration in the final mixture indicated that tannins were absent.

****Test for Carbohydrates:****

Molisch Test:

In this procedure, 2 milliliters of concentrated sulfuric acid were added to a 2 milliliter aliquot of the extract solution. Following this, it was treated with 15% ethanolic α-naphthol, known as Molisch's reagent. The formation of a red-violet ring where the two layers met confirmed the presence of gums, signifying a positive result for the test.

Fehling's Test:

Here, 5 milliliters of the extract solution were combined with an equal volume of Fehling's solution and heated for five minutes. The emergence of a brick-red precipitate in the mixture signaled a positive result for reducing sugars.

Benedict's Test:

In this test, 5 milliliters of the extract solution were added to 5 milliliters of Benedict's solution in a test tube and boiled for five minutes. The appearance of a brick-red precipitate confirmed the presence of reducing sugars.

Anthrone Test:

For the Anthrone test, 5 milliliters of the extract solution were mixed with 10 milliliters of water and shaken well. The solution was then filtered, and the filtrate was concentrated with the addition of 2 milliliters of Anthrone reagent. A greenish-blue color developed, confirming the presence of reducing sugars.

****Test for Glycosides:****

Legal's Test:

The extract solution was dissolved in pyridine and mixed with sodium nitroprusside solution. The appearance of a red color indicated the presence of glycosides.

Borntrager's Test:

In this test, the extract solution was boiled with 1 milliliter of concentrated sulfuric acid. After cooling, the solution was filtered, and to the filtrate, equal volumes of chloroform and ammonia were added. The resulting appearance of a red-pink color confirmed the presence of glycosides.

Baljet's Test:

To test for glycosides, picric acid was added to 2 milliliters of the test solution (the extract). The formation of an orange-red color signified the presence of glycosides.

****Test for Alkaloids:****

Fifty milligrams of the extract were stirred with a few milliliters of dilute hydrochloric acid, followed by filtration.

Mayer's Test:

The resulting filtrate was placed in a test tube and treated with 0.1 milliliters of Mayer's reagent. The formation of a yellowish buff precipitate indicated a negative result for alkaloids.

Wagner's Test:

In this test, the filtrate was treated with 0.1 milliliters of Wagner's reagent. The absence of a reddish-brown precipitate indicated a negative result for alkaloids.

Hager's Test:

The filtrate was subjected to a few drops of Hager's reagent. The lack of any yellow precipitate indicated that alkaloids were not present.

Dragendorff's Test:

For this test, the filtrate was treated with several drops of Dragendorff's reagent. The absence of an orange-red precipitate further confirmed the lack of alkaloids.

****Test for Resins:****

One milliliter of the test solution (the extract) was mixed with acetone and poured into distilled water. The turbidity of the resulting solution was assessed, indicating the absence of resins.

RESULTS:

The plant material underwent a thorough authentication process conducted by the esteemed Botanical Survey of India in Gangtok, Sikkim. This process culminated in issuing a certificate bearing the reference number SHRC-5/02/2023-24/Tech/272. This certificate has been securely filed at the School of Natural Product Studies to ensure its safekeeping, specifically within the Department of Pharmaceutical Technology. This department is part of Jadavpur University, which is located in the vibrant city of Kolkata, West Bengal, India.

The fresh rhizomes and roots of Zingiber rubens were meticulously gathered and dried to preserve their essential properties. Once dried, the materials were ground into smaller fragments to enhance the extraction process, and then sifted through a fine mesh with an aperture size of 100 to achieve a uniform particle size. The resulting crude powders were carefully packed into a thimble and placed inside a Soxhlet apparatus, filled with a carefully prepared hydroalcoholic solvent mixture of 70% methanol and 30% water. This setup allowed for efficient extraction of the active compounds. After the extraction process was complete, the hydroalcoholic extract of Zingiber rubens (HAZR) was collected, gently dried to remove any residual moisture, and then stored in a sealed airtight container at a cool temperature of 4°C to ensure its stability and integrity for future experimental use. The % yield was found as 11.25 % W/W.

Sl. No.	Constituents	F-1:N-hexane
1	*Flavonoids*	+ve
2	*Phenolic Compounds*	+ve
3	*Steroids*	+ve
4	*Saponins*	+ve
5	*Glycosides*	+ve
6	*Carbohydrates*	+ve

Table 01: Qualitative phytochemical test.

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Chemicals:

The following reagents were utilized in this study: 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and dithiopenta acetic acid (DTPA) were sourced from Sigma Chemicals, a reputable supplier based in the USA. Additionally, several other crucial chemicals were obtained from Sisco Research Laboratories Pvt. Ltd., located in Mumbai, India. These include nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), and reduced nicotinamide adenine dinucleotide (NADH), which play significant roles in various biochemical assays.

Moreover, sodium nitroprusside, naphthyl ethylene diamine dihydrochloride, and ascorbic acid were included for their contributions to redox reactions and antioxidant studies. Trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were also used for analyzing lipid peroxidation, while ethylene diamine tetra acetic acid (EDTA) served as a chelating agent. Other reagents, including sodium hydroxide (NaOH), hydrogen peroxide (H2O2), and butylated hydroxy anisole (BHA), were selected for their antioxidant properties. Furthermore, deoxyribose and potassium ferricyanide [K3Fe(CN)6] were included for their roles in spectrophotometric assays, and Folin-Ciocalteu's phenol reagent (FCR) was used for assessing phenolic content. All chemicals employed were of high analytical grade to ensure the accuracy and reliability of the experimental results.

DPPH radical scavenging activity [1-5]:

The DPPH radical scavenging activity was assessed utilizing a method outlined in previous studies [7], with some modifications for enhanced accuracy. In this procedure, a minimum of 1 ml of DPPH solution, prepared at a concentration of 100 µM in methanol, was combined with 1 ml of the test fractions, which were tested at various concentrations. This mixture was then incubated at a controlled temperature of 37 °C for a period of 30 minutes to allow for adequate interaction between the DPPH radicals and the test samples. Following the incubation, the absorbance of the resultant solution was measured at a wavelength of 517 nm using a spectrophotometer, a critical step for quantifying the degree of radical scavenging. To determine the percentage inhibition of DPPH radicals, the absorbance readings from the test samples were compared against those of the control group, which was not treated with any fractions, employing a specific calculation formula for this purpose.

Percentage inhibition= [(CON-TEST)/CON] × 100, Where, CON = Absorbance at 517 nm of the control and TEST = Absorbance at 517 nm of the test

Nitric oxide scavenging activity [2]:

In an aqueous solution at physiological pH, sodium nitroprusside serves as a precursor for the generation of nitric oxide. This nitric oxide subsequently interacts with oxygen, leading to the formation of nitrite ions, which can be quantitatively assessed through the Griess reaction (Bala et al., 2009; Kundu et al., 2011).

The experimental procedure begins with the careful mixing of 0.1 ml of a 10 mM sodium nitroprusside solution with 1 ml of either the test solution or standard ascorbic acid at varying concentrations. This mixture is prepared within a phosphate buffer of 100 mM at a pH of 7.4. Following the mixing, the resulting solution is incubated at a controlled temperature of 25°C for a duration of 150 minutes to allow for the reactions to proceed.

After the incubation period, a 1 ml aliquot of this mixture is taken and combined with 1 ml of Griess reagent. The Griess reagent is prepared by mixing equal parts of a 0.1% naphthyl ethylene diamine dihydrochloride solution in water and a 1% sulfanilamide solution in 2% o-phosphoric acid. This combined solution is then allowed to stand in the dark at room temperature for 10 minutes, facilitating the development of the chromophore.

The resulting absorbance, which is indicative of the nitrite concentration formed through the diazotization reaction with sulfanilamide and its subsequent coupling with naphthyl ethylene diamine dihydrochloride, is measured at a wavelength of 546 nm. Finally, the percentage of inhibition is calculated by comparing the experimental results with those from a control, using the previously mentioned analytical formula to quantify the effect of the test substances.

Superoxide radical scavenging activity [3]:

The scavenging activity of superoxide anions was assessed using a modified version of a previously described method. All solutions were meticulously prepared in a 100 mM phosphate buffer, ensuring a pH of 7.4 for optimal reaction conditions. The reaction mixture consisted of 1 ml of nitroblue tetrazolium (NBT) solution at a concentration of 156 µM, combined with 1 ml of reduced nicotinamide adenine dinucleotide (NADH) at 468 µM. To this, 3 ml of either the test solution or a standard solution of ascorbic acid was added, with varying concentrations employed for comparison.

The reaction was initiated by the addition of 100 µl of phenazine methosulfate (PMS) at a concentration of 60 µM and was allowed to incubate at a constant temperature of 25° C for a duration of 5 minutes. Following the incubation period, the absorbance of the resulting mixture was measured at a wavelength of 560 nm. The percentage of superoxide anion inhibition was subsequently calculated using the specified formula, allowing for an evaluation of the scavenging effectiveness of the test substances.

Hydroxyl radical scavenging activity [3-5]:

The scavenging capacity for hydroxyl radicals was assessed using a modified method described in previous research. To begin the experiment, stock solutions of various reagents were prepared in distilled deionized water: EDTA at a concentration of 1 mM, FeCl3 at 10 mM, ascorbic acid at 1 mM, hydrogen peroxide (H2O2) at 10 mM, and deoxyribose at 10 mM.

The assay itself involved several steps. First, a precise volume of each solution was combined: 0.1 ml of EDTA, followed by 0.01 ml of FeCl3, and then 0.1 ml of H2O2. Next, 0.36 ml of deoxyribose was added, along with 1.0 ml of either the extract being tested or standard ascorbic acid solutions at various concentrations. To ensure optimal reaction conditions, 0.33 ml of a phosphate buffer (50 mM, pH 7.4) was introduced, along with 0.1 ml of ascorbic acid. This sequence of additions was crucial for the subsequent chemical reactions.

The mixture was then incubated at a controlled temperature of 37°C for one hour, allowing sufficient time for the reactions to occur. Following incubation, about 1 ml of the resulting mixture was transferred to a new container, where it was combined with 1 ml of trichloroacetic acid (TCA) and 1 ml of a 0.5% thiobarbituric acid (TBA) solution, which was prepared in 0.025 M sodium hydroxide (NaOH) and contained 0.025% butylated hydroxyanisole (BHA). This step was essential for developing the pink chromogen, which would later be quantified.

Finally, the absorbance of the developed chromogen was measured at a wavelength of 532 nm using a spectrophotometer. The effectiveness of the extract in scavenging hydroxyl radicals was expressed as the percentage inhibition of deoxyribose degradation, which was calculated according to previously established methods. This detailed approach allows for a clear evaluation of the antioxidant capacity of the extracts being studied.

Data analysis: All data are presented as the mean ± SEM from three individual measurements. The 50% inhibitory concentrations (IC50) were determined by plotting the data on a graph with concentration versus percentage inhibition, using GraphPad Prism software, version 4.03.

RESULTS:

The antioxidant potential of HAZR was evaluated through the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, with ascorbic acid serving as the reference standard. The results, depicted in Figure 1, illustrate the percentage inhibition of DPPH activity relative to varying concentrations of the extract. HAZR demonstrated noteworthy effectiveness in scavenging free radicals, exhibiting an IC50 value of 25 µg/ml, in contrast to the standard ascorbic acid, which recorded an IC50 of approximately 42 µg/ml.

Additionally, the scavenging activity against hydroxyl radicals was assessed using a reaction mixture that included 2-deoxy-D-ribose, the extract, EDTA, thio-barbituric acid, and trichloroacetic acid, again employing ascorbic acid as the standard. HAZR showed a remarkable inhibitory half-maximal concentration (IC50) of 23 µg/ml, significantly lower than the IC50 of about 39 µg/ml observed for the standard ascorbic acid, as illustrated in Figure 1.

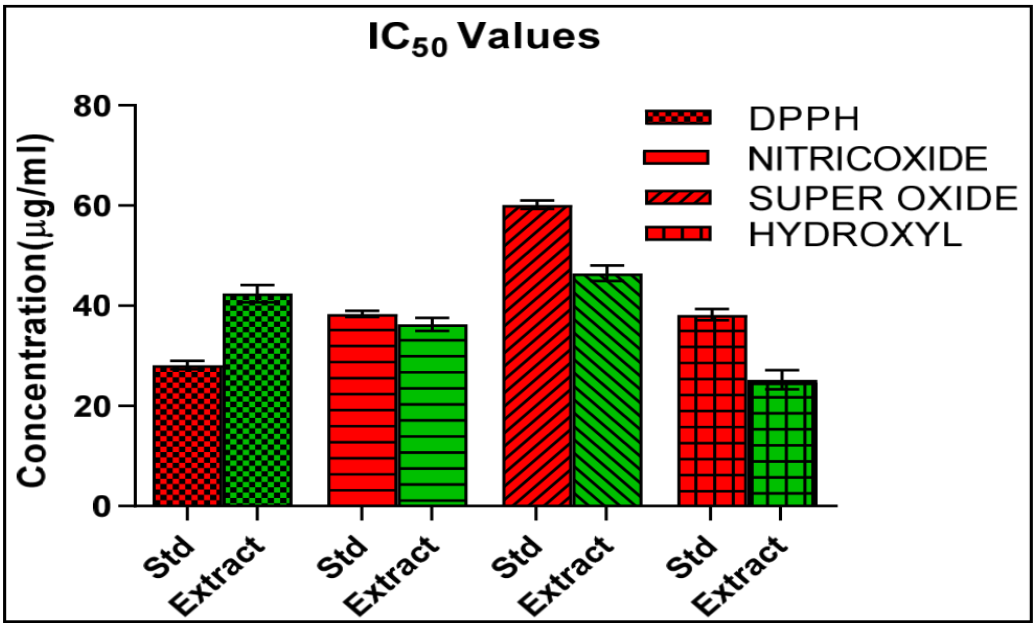

Figure 01.: Comparative IC50 values of in vitro free radical scavenging assay. Values are expressed as mean ±SEM (n=3), and Vitamin C was used as standard (Std).

To evaluate the inhibition of nitric oxide radicals, a range of concentrations (0.2–0.8 mg/mL) of the extract was tested using Griess reagent. The percentage inhibition of nitric oxide was calculated and graphed against concentration, shown in Supplementary Figure 2. HAZR proved to be effective, yielding an IC50 value of 38 µg/ml, which indicates its substantial activity relative to the standard.

Furthermore, the superoxide anion scavenging ability of HAZR was examined using nitro-blue tetrazolium (NBT) in a Tris-HCl buffer, initiated by a phenazine methosulfate (PMS) solution. The results revealed that HAZR acted as a potent antioxidant with an IC50 value of 45 µg/ml, outperforming the widely used ascorbic acid standard, which yielded an IC50 value of 60 µg/ml, as represented in Figure 1.

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α-AMYLASE INHIBITORY ACTIVITY [1]:

This activity was conducted following the methodology established by Jana et al., 2023 [1], with some minor modifications. Acarbose served as the standard control for this experiment. To prepare the starch solution, 1 gram of starch was meticulously dissolved in 50 milliliters of sodium hydroxide (NaOH) and then heated to a boiling point of 100°C for 5 minutes, ensuring thorough dissolution. Following this heating process, the solution was rapidly cooled using ice-cold water. The pH of the solution was subsequently adjusted to a neutral value of 7 by carefully adding 2M hydrochloric acid (HCl). Finally, the solution's volume was increased to 100 milliliters by adding distilled water.

For sample preparation, 10 milligrams of the extract were combined with 1 milliliter of a methanol-water (1:1) solution. To ensure complete enzyme inhibition, dilutions of phosphate-buffered saline (PBS) were created by mixing 35 microliters of PBS, 35 microliters of the starch substrate, and 5 microliters of the prepared sample in a 96-well microtiter plate. The plate was then incubated at 37°C for 1 minute to allow the components to interact. After this initial incubation, 20 microliters of a 50 mg/mL α-Amylase solution were carefully added to each well, and the reaction was allowed to proceed for 15 minutes. The reaction was terminated by adding 50 microliters of 0.1 M HCl, followed by the addition of 150 microliters of a 0.5 mM iodine solution to visualize the starch digestion.. The absorbance was measured at 580 nm. % inhibition of α-Amylase = [1 − (ABS2 − ABS1) / (ABS4 − ABS3) × 100]

Here, ABS_1: absorbance of incubated solution containing sample, starch and amylase

ABS_2: absorbance of incubated solution containing sample and starch

ABS_3: absorbance of an incubated solution containing starch and amylase, and

ABS_4: absorbance of an incubated solution containing starch

IC_{50} value was calculated by interpolating using Graph Pad Prism software version 5 considering force zero (X=0 and Y=0)

α-GLUCOSIDASE INHIBITION ASSAY [2-4]:

To assess the enzymatic activity, a 96-well microtiter plate was employed as the experimental setup. The substrate utilized in this analysis was p-nitrophenyl-α-D-glucopyranoside, a compound that facilitates the measurement of α-glucosidase activity upon hydrolysis. The

experimental procedure was adapted from the method described by Jana et al. in 2023 [1], incorporating minor adjustments to optimize the results.

To prepare the samples for testing, 10 mg of the extract was dissolved in 1 mL of a methanol-water mixture in a 1:1 ratio, resulting in a homogenous solution. Sequential dilutions of phosphate-buffered saline (PBS) were prepared to ensure appropriate conditions for the reaction. The enzymatic reaction itself began by mixing 75 µL of the prepared PBS with 5 µL of the sample solution and 20 µL of the enzyme solution. This mixture was pre-incubated at a controlled temperature of 37 °C for a duration of 5 minutes to foster optimal conditions for the enzyme's activity before the reaction commenced.

The reaction was initiated by the introduction of the substrate, p-nitrophenyl-α-D-glucopyranoside. To monitor the progress of the reaction, the release of p-nitrophenol (p-NP) was measured over the course of one hour using a microplate reader set at an absorbance wavelength of 405 nm. Absorbance readings were recorded at 5-minute intervals to track the reaction kinetics continuously. Acarbose was used as the standard control to compare the inhibitory effects of the extracts on α-glucosidase activity. The percentage inhibition of α-glucosidase was calculated using the formula: % inhibition = [(A0 − AS) / A0] × 100, where A0 represents the absorbance in the absence of the inhibitor, and AS corresponds to the absorbance in the presence of the extract.Here, A_0: absorbance recorded for the control (enzymatic activity without inhibitor); A_S: absorbance recorded for the sample (enzymatic activity in the presence of the inhibitor). IC_{50} value was calculated by interpolating using Graph Pad prism software version 5 considering force zero (X=0 and Y=0)

Data analysis: All data are presented as the mean value along with the standard error of the mean (SEM), which was derived from three separate measurements. To determine the 50% inhibitory concentrations (IC50), we plotted the concentration of the substance against the percentage of inhibition observed. This analysis was conducted using GraphPad Prism software, version 4.03, allowing for precise visualization and interpretation of the data.

RESULTS:

The in vitro inhibitory activity of HAZR against the enzyme α-amylase was evaluated by determining the half-maximal inhibitory concentration (IC50) at varying concentrations of the plant extract, using the established standard marker, Acarbose, for comparison. The analysis revealed that the IC50 value for HAZR was recorded at 373.6 ± 2.27 µg/ml, while the standard

drug Acarbose exhibited an IC50 of 317.1 ± 3.32 µg/ml. This indicates that the inhibitory activity of HAZR is nearly comparable to that of Acarbose, as illustrated in Figure 1 .

Furthermore, the inhibitory effect of HAZR on α-glucosidase was similarly assessed by calculating the IC50 value at multiple concentrations of the plant extract, again using Acarbose as the benchmark inhibitor. In this case, the IC50 value for HAZR was determined to be 345.6 ± 5.63 µg/ml, in stark contrast to Acarbose, which demonstrated a significantly lower IC50 of 51.2 ± 1.63 µg/ml. These results highlight the differential effectiveness of HAZR in inhibiting α-glucosidase compared to the standard inhibitor.

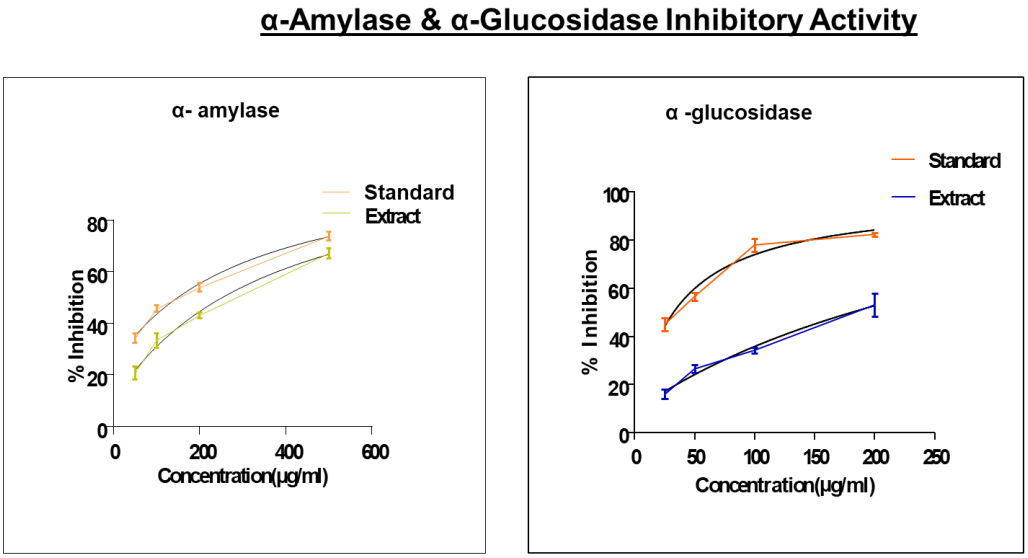

Figure 01: % inhibition of α-amylase shown by different concentrations of Extract and Acarbose. A. The IC_{50} value for α-amylase was 373.6 ± 2.27 µg/ml and 317.1 ± 3.32 µg/ml for the HAZR and the standard drug Acarbose, respectively. B. The IC_{50} value for α-glucosidase was 345.6 ± 5.63 µg/ml, and 51.2 ± 1.63 µg/ml for the HAZR and the standard drug Acarbose, respectively.

References:

[1.]Gayen S, Jana S, Das Gupta B, Ghosh A, Kar A, Bala A, Mukherjee PK, Haldar PK. Exploration of anti-diabetic activity and metabolite profiling of Bruguiera cylindrica (l.) Bl.-in vivo anti-diabetic activity, exploration of molecular mechanism, and network pharmacological analysis. J Pharm Pharmacol. 2024 Jul 5;76(7):798-812.

[2.] Jana S, Gayen S, Gupta BD, Singha S, Mondal J, Kar A, Nepal A, Ghosh S, Rajabalaya R, David SR, Balaraman AK, Bala A, Mukherjee PK, Haldar PK. Investigation on Anti-diabetic Efficacy of a Cucurbitaceae Food Plant from the North-East Region of India: Exploring the Molecular Mechanism through Modulation of Oxidative Stress and Glycosylated Hemoglobin (HbA1c). Endocr Metab Immune Disord Drug Targets. 2024;24(2):220-234. doi: 10.2174/1871530323666230907115818.

[3.] Naskar S, Mazumder UK, Pramanik G, Gupta M, Kumar RB, Bala A, Islam A. Evaluation of antihyperglycemic activity of Cocos nucifera Linn. on streptozotocin induced type 2 diabetic rats. J Ethnopharmacol. 2011 Dec 8;138(3):769-73.

[4.] Patra S, Bhattacharya S, Bala A, Haldar PK. Antidiabetic effect of Drymaria cordata leaf against streptozotocin-nicotinamide-induced diabetic albino rats. J Adv Pharm Technol Res. 2020 Jan-Mar;11(1):44-52. doi: 10.4103/japtr.JAPTR_98_19.

Network Pharmacological Analysis:

Network pharmacology represents an innovative research approach that merges the principles of systems network analysis with the field of pharmacology. This methodology aims to uncover the intricate mechanisms through which multi-target drugs operate at the molecular level in the treatment of various diseases. By employing comprehensive analyses of compound-target interactions as well as the relationships between targets and diseases, network pharmacology enables researchers to better understand how different drugs can simultaneously affect multiple biological pathways, thereby enhancing therapeutic efficacy and advancing personalized medicine [1].

Methodology [2-5]:

On August 30, 2023, a comprehensive effort was undertaken to download a total of 21 publications from the PUBMED database, focusing on the keywords “Stevia rebaudiana (Bertoni) Bertoni.” Each of these publications underwent meticulous examination to identify unique phytochemicals that have been experimentally validated in Stevia rebaudiana. The chemical structures and molecular formulas of these compounds were rigorously cross-verified utilizing the PubChem database, ensuring accuracy and reliability.

To delve deeper into the biological significance of these compounds, their structures were exported in SDF (Structure Data File) format and then submitted to the Binding Database (https://www.bindingdb.org/rwd/bind/index.jsp) for analysis of potential biological targets. This search was narrowed down to human protein targets that demonstrated a high degree of similarity, specifically a threshold of ≥ 0.7. A schematic overview of this intricate methodology is illustrated in Fig. 1, providing clarity on the steps taken.

The identified protein targets were further investigated to ascertain their corresponding gene names through the UniProt database. These gene names were then reformatted into a double annotated gene format, enabling a search in the DisGeNET database version 7.0 to uncover gene-disease associations relevant to various health conditions.

To synthesize these findings into a coherent representation, a compound-disease-target network was constructed and analyzed using CYTOSCAPE version 3.10.0, facilitating a deeper understanding of the interplay between the phytochemicals of Stevia rebaudiana

and their potential implications in disease contexts (Gayen et al., 2024).

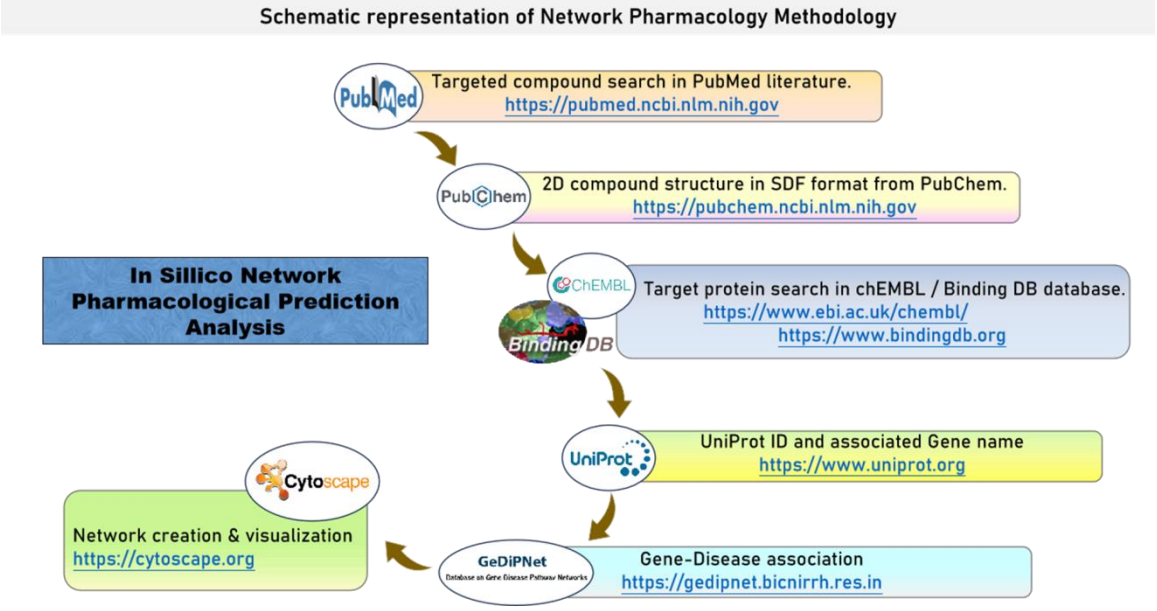

Figure 1: Methodology opted for network pharmacological analysis.

Sl. No.	Plant compound	PubChem compound ID	Canonical SMILES
1	(Z)-citral	643779	CC(=CCCC(=CC=O)C)C
2	camphene	6616	CC1(C2CCC(C2)C1=C)C
3	β-phellandrene	11142	CC(C)C1CCC(=C)C=C1
4	1,8-cineole	2758	CC1(C2CCC(O1)(CC2)C)C
5	zingiberene	92776	CC1=CCC(C=C1)C(C)CCC=C(C)C
6	Geraniol	637566	CC(=CCCC(=CCO)C)C

Table 1: Targeted Plant compound from *Zingiber rubens*

Compound	Target protein	UniProt ID	Gene name
(Z)-citral	Cathepsin Z	Q9UBR2	CTSZ
(Z)-citral	Proteasome subunit beta type-7	Q99436	PSMB7
(Z)-citral	Receptor-type tyrosine-protein phosphatase zeta	P23471	PTPRZ1
(Z)-citral	Thioredoxin reductase 2, mitochondrial	Q9NNW7	TXNRD2
(Z)-citral	Dual specificity protein phosphatase 23	Q9BVJ7	DUSP23
(Z)-citral	Alpha-2-HS-glycoprotein	P02765	AHSG
(Z)-citral	Calpain-1 catalytic subunit	P07384	CAPN1
(Z)-citral	Calpain-2 catalytic subunit	P17655	CAPN2
(Z)-citral	Calpain small subunit 1	P04632	CAPNS1
(Z)-citral	Thioredoxin reductase 3	Q86VQ6	TXNRD3
(Z)-citral	Thioredoxin reductase 2, mitochondrial	Q9NNW7	TXNRD2
(Z)-citral	Thioredoxin reductase 1, cytoplasmic	Q16881	TXNRD1
(Z)-citral	Proteasome subunit beta type-1	P20618	PSMB1
(Z)-citral	Proteasome subunit alpha type-7	O14818	PSMA7
(Z)-citral	Proteasome subunit alpha type-2	P25787	PSMA2
(Z)-citral	Proteasome subunit beta type-3	P49720	PSMB3
(Z)-citral	Proteasome subunit beta type-7	Q99436	PSMB7
(Z)-citral	Proteasome subunit alpha type-1	P25786	PSMA1
(Z)-citral	Proteasome subunit beta type-4	P28070	PSMB4
(Z)-citral	Proteasome subunit beta type-2	P49721	PSMB2
(Z)-citral	Proteasome subunit alpha type-6	P60900	PSMA6
(Z)-citral	Proteasome subunit beta type-9	P28065	PSMB9
(Z)-citral	Proteasome subunit beta type-6	P28072	PSMB6
(Z)-citral	Proteasome subunit beta type-5	P28074	PSMB5
(Z)-citral	Proteasome subunit beta type-11	A5LHX3	PSMB11
(Z)-citral	Proteasome subunit alpha type-5	P28066	PSMA5
(Z)-citral	Proteasome subunit beta type-10	P40306	PSMB10
(Z)-citral	Proteasome subunit alpha type-3	P25788	PSMA3
(Z)-citral	Proteasome subunit alpha type-4	P25789	PSMA4
(Z)-citral	Proteasome subunit beta type-8	P28062	PSMB8
(Z)-citral	Proteasome subunit alpha-type 8	Q8TAA3	PSMA8
(Z)-citral	26S proteasome non-ATPase regulatory subunit 14	O00487	PSMD14
(Z)-citral	26S proteasome non-ATPase regulatory subunit 4	P55036	PSMD4
(Z)-citral	26S proteasome non-ATPase regulatory subunit 7	P51665	PSMD7
(Z)-citral	26S proteasome complex subunit SEM1	P60896	SEM1
(Z)-citral	Proteasomal ubiquitin receptor ADRM1	Q16186	ADRM1
(Z)-citral	26S proteasome non-ATPase regulatory subunit 1	Q99460	PSMD1
(Z)-citral	26S proteasome non-ATPase regulatory subunit 12	O00232	PSMD12
(Z)-citral	26S proteasome regulatory subunit 7	P35998	PSMC2
(Z)-citral	26S proteasome regulatory subunit 6B	P43686	PSMC4

(Z)-citral	26S proteasome regulatory subunit 10B	P62333	PSMC6
(Z)-citral	26S proteasome non-ATPase regulatory subunit 3	O43242	PSMD3
(Z)-citral	26S proteasome regulatory subunit 6A	P17980	PSMC3
(Z)-citral	26S proteasome non-ATPase regulatory subunit 8	P48556	PSMD8
(Z)-citral	26S proteasome regulatory subunit 4	P62191	PSMC1
(Z)-citral	26S proteasome non-ATPase regulatory subunit 2	Q13200	PSMD2
(Z)-citral	26S proteasome non-ATPase regulatory subunit 13	Q9UNM6	PSMD13
(Z)-citral	26S proteasome non-ATPase regulatory subunit 11	O00231	PSMD11
(Z)-citral	26S proteasome regulatory subunit 8	P62195	PSMC5
(Z)-citral	26S proteasome non-ATPase regulatory subunit 6	Q15008	PSMD6
Camphene	Carbonic anhydrase II	P00918	CA2
Camphene	Beta amyloid A4 protein	E9PG40	APP
Camphene	Neuronal acetylcholine receptor; alpha3	P09481	CHRNA3
Camphene	Neuronal acetylcholine receptor subunit alpha-4	P09482	CHRNA4
Camphene	Neuronal acetylcholine receptor beta4	P30926	CHRNB4
Camphene	Indoleamine 2,3-dioxygenase	P14902	IDO1
Camphene	Sodium channel protein type III alpha subunit	Q9NY46	SCN3A
Camphene	Dihydrofolate reductase	P00374	DHFR
Camphene	Adenosine deaminase	P00813	ADA
Camphene	Prostanoid EP4 receptor	P35408	PTGER4
Camphene	Sodium/hydrogen exchanger 1	P19634	SLC9A1
Camphene	Serotonin 3a (5-HT3a) receptor	P46098	HTR3A
Camphene	Adenosine A1 receptor	P30542	ADORA1
Camphene	Monoglyceride lipase	Q99685	MGLL
Camphene	Tyrosine-protein kinase SRC	P12931	SRC
Camphene	Protein farnesyltransferase	P49356	FNTB
Camphene	Muscarinic acetylcholine receptor M1	P11229	CHRM1
Camphene	Glutamate NMDA receptor GRIN1	Q05586	GRIN1
Camphene	Glutamate NMDA receptor GRIN2A	Q12879	GRIN2A
Camphene	Glutamate NMDA receptor GRIN2B	Q13224	GRIN2B
Camphene	LSD1/CoREST complex	O60341	KDM1A
Camphene	Sodium/glucose cotransporter 1	P13866	SLC5A1
Camphene	T-cell protein-tyrosine phosphatase	P17706	PTPN2
1,8-cineole	Carbonic anhydrase II	P00918	CA2
1,8-cineole	Beta amyloid A4 protein	E9PG40	APP
1,8-cineole	Neuronal acetylcholine receptor alpha3	P09481	CHRNA3
1,8-cineole	Neuronal acetylcholine receptor beta4	P30926	CHRNB4
1,8-cineole	Heat shock protein HSP 90-alpha	P07900	HSP90AA1
1,8-cineole	Dihydrofolate reductase	P00374	DHFR
1,8-cineole	Adenosine deaminase	P00813	ADA
1,8-cineole	Prostanoid EP4 receptor	P35408	PTGER4
1,8-cineole	Sodium/hydrogen exchanger 1	P19634	SLC9A1

1,8-cineole	Adenosine A1 receptor	P30542	ADORA1
1,8-cineole	Acyl coenzyme A:cholesterol acyltransferase 2	Q9BWD1	ACAT2
1,8-cineole	Serotonin 3a (5-HT3a) receptor	P46098	HTR3A
1,8-cineole	Monoglyceride lipase	Q99685	MGLL
1,8-cineole	Tyrosine-protein kinase SRC	P12931	SRC
1,8-cineole	Type-1A angiotensin II receptor	P30556	AGTR1
1,8-cineole	Tryptase beta-1	Q15661	TPSAB1
1,8-cineole	Glutamate NMDA receptor GRIN1	Q05586	GRIN1
1,8-cineole	Glutamate NMDA receptor GRIN2A	Q12879	GRIN2A
1,8-cineole	Glutamate NMDA receptor GRIN2B	Q13224	GRIN2B
1,8-cineole	LSD1/CoREST complex	O60341	KDM1A
1,8-cineole	Sodium/glucose cotransporter 1	P13866	SLC5A1
zingiberene	Adenosine receptor A1	P30542	ADORA1
zingiberene	Adenosine receptor A3	P0DMS8	ADORA3
zingiberene	Adenosine receptor A2a	P29274	ADORA2A
zingiberene	Alpha-synuclein	P37840	SNCA
zingiberene	Amyloid-beta precursor protein	P05067	APP
zingiberene	Solute carrier organic anion transporter family member 1B1	Q9Y6L6	SLCO1B1
Geraniol	Amine oxidase [flavin-containing] B	P27338	MAOB
β-phellandrene	Alpha-synuclein	P37840	SNCA
β-phellandrene	Amyloid-beta precursor protein	P05067	APP
β-phellandrene	Solute carrier organic anion transporter family member 1B1	Q9Y6L6	SLCO1B1

Table 2: Compound target protein and gene name for analysis.

Compound	Target protein	UniProt ID	Gene name
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(Z)-citral	**Proteasome subunit beta type-7**	Q99436	**PSMB7**
(Z)-citral	**Receptor-type tyrosine-protein phosphatase zeta**	P23471	**PTPRZ1**
(Z)-citral	**Thioredoxin reductase 2, mitochondrial**	Q9NNW7	**TXNRD2**
(Z)-citral	**Dual specificity protein phosphatase 23**	Q9BVJ7	**DUSP23**
(Z)-citral	**Alpha-2-HS-glycoprotein**	P02765	**AHSG**
(Z)-citral	**Calpain-1 catalytic subunit**	P07384	**CAPN1**
(Z)-citral	**Calpain-2 catalytic subunit**	P17655	**CAPN2**
(Z)-citral	**Calpain small subunit 1**	P04632	**CAPNS1**
(Z)-citral	**Thioredoxin reductase 3**	Q86VQ6	**TXNRD3**
(Z)-citral	**Thioredoxin reductase 2, mitochondrial**	Q9NNW7	**TXNRD2**
(Z)-citral	**Thioredoxin reductase 1, cytoplasmic**	Q16881	**TXNRD1**
(Z)-citral	**Proteasome subunit beta type-1**	P20618	**PSMB1**

(Z)-citral	**Proteasome subunit alpha type-7**	O14818	**PSMA7**
(Z)-citral	**Proteasome subunit alpha type-2**	P25787	**PSMA2**
(Z)-citral	**Proteasome subunit beta type-3**	P49720	**PSMB3**
(Z)-citral	**Proteasome subunit beta type-7**	Q99436	**PSMB7**
(Z)-citral	**Proteasome subunit alpha type-1**	P25786	**PSMA1**
(Z)-citral	**Proteasome subunit beta type-4**	P28070	**PSMB4**
(Z)-citral	**Proteasome subunit beta type-2**	P49721	**PSMB2**
(Z)-citral	**Proteasome subunit alpha type-6**	P60900	**PSMA6**
(Z)-citral	**Proteasome subunit beta type-9**	P28065	**PSMB9**
(Z)-citral	**Proteasome subunit beta type-6**	P28072	**PSMB6**
(Z)-citral	**Proteasome subunit beta type-5**	P28074	**PSMB5**
(Z)-citral	**Proteasome subunit beta type-11**	A5LHX3	**PSMB11**
(Z)-citral	**Proteasome subunit alpha type-5**	P28066	**PSMA5**
(Z)-citral	**Proteasome subunit beta type-10**	P40306	**PSMB10**
(Z)-citral	**Proteasome subunit alpha type-3**	P25788	**PSMA3**
(Z)-citral	**Proteasome subunit alpha type-4**	P25789	**PSMA4**
(Z)-citral	**Proteasome subunit beta type-8**	P28062	**PSMB8**
(Z)-citral	**Proteasome subunit alpha-type 8**	Q8TAA3	**PSMA8**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 14**	O00487	**PSMD14**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 4**	P55036	**PSMD4**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 7**	P51665	**PSMD7**
(Z)-citral	**26S proteasome complex subunit SEM1**	P60896	**SEM1**
(Z)-citral	**Proteasomal ubiquitin receptor ADRM1**	Q16186	**ADRM1**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 1**	Q99460	**PSMD1**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 12**	O00232	**PSMD12**
(Z)-citral	**26S proteasome regulatory subunit 7**	P35998	**PSMC2**
(Z)-citral	**26S proteasome regulatory subunit 6B**	P43686	**PSMC4**
(Z)-citral	**26S proteasome regulatory subunit 10B**	P62333	**PSMC6**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 3**	O43242	**PSMD3**
(Z)-citral	**26S proteasome regulatory subunit 6A**	P17980	**PSMC3**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 8**	P48556	**PSMD8**
(Z)-citral	**26S proteasome regulatory subunit 4**	P62191	**PSMC1**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 2**	Q13200	**PSMD2**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 13**	Q9UNM6	**PSMD13**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 11**	O00231	**PSMD11**

(Z)-citral	**26S proteasome regulatory subunit 8**	P62195	**PSMC5**
(Z)-citral	**26S proteasome non-ATPase regulatory subunit 6**	Q15008	**PSMD6**

Table 3: Compound target protein and gene name for analysis.

RESULTS:

The analysis of the selected seven compounds has been thoroughly conducted, focusing on their interactions within a comprehensive network of 297 targeted proteins and enzymes. This intricate network was visualized using CYTOSCAPE, a powerful tool for biological network analysis. In this study, the 32 chosen compounds were represented within the network by solid lines, illustrating the various interconnections among them. Notably, the compounds that exhibited the highest degree of interaction with these proteins were marked within a red dotted circle, emphasizing their significance in the network. It was observed that most isolated compounds primarily interacted with three key proteins: the 5′-AMP-activated protein kinase catalytic subunit alpha-2/β1/γ1, 1,3-beta-D-glucan synthase, and Xanthine dehydrogenase/oxidase.

Further analysis of the interactions was performed using tools within CYTOSCAPE, leading to the creation of a degree layout plotted against the respective compounds, as illustrated in Fig. 3. In network terminology, the degree of a node is defined by the number of connections (or edges) it has to other nodes. In the context of protein-drug interaction networks, nodes with a greater number of connections are identified as hubs. These hubs are vital for the overall structure and functional integrity of the network, playing an essential role in mediating biological processes and responses.

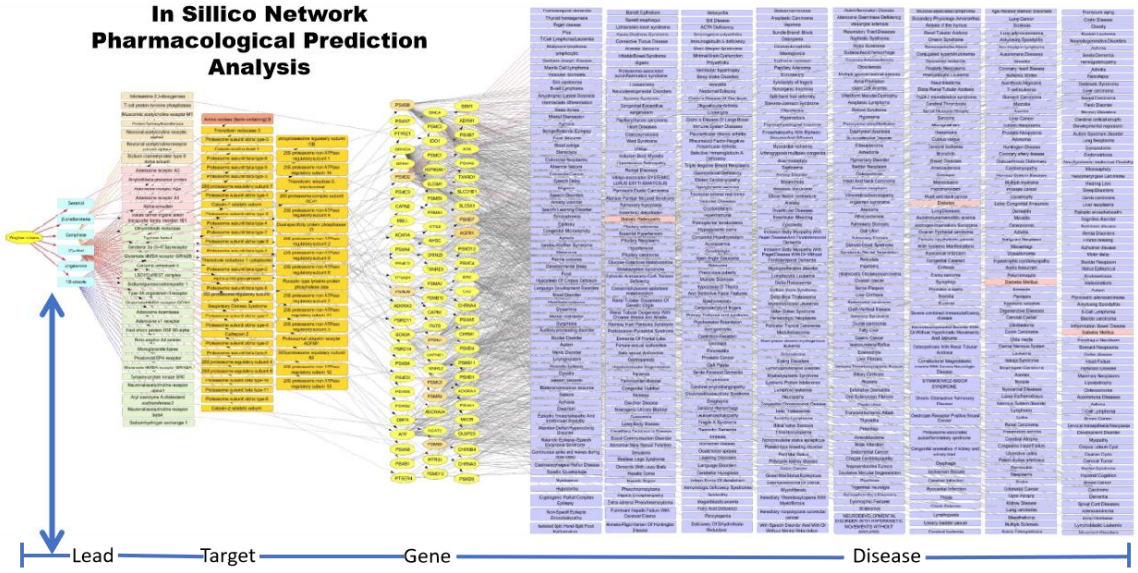

Figure 2: Gene Disease Association of *Zingiber rubens* – phytochemicals.

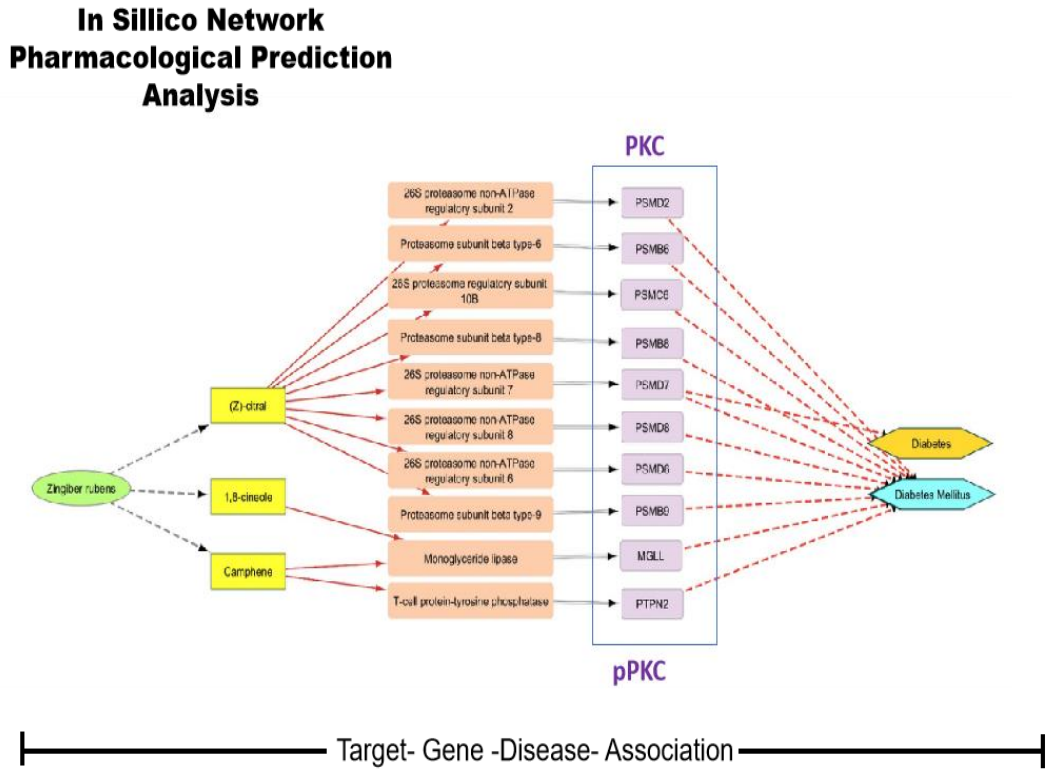

Figure 2: Gene Disease Association of *Zingiber rubens* – phytochemicals. The Associated protein targeted is- Protein Kinase C.

CAPNS1 Prostatic Neoplasms
CAPNS2 Prostate cancer
TXNRD2 Anorexia
TXNRD2 Azoospermia
TXNRD2 Cardiomyopathy
TXNRD2 Congenital Hypothyroidism
TXNRD2 Cryptorchidism
TXNRD2 Dilated Cardiomyopathy
TXNRD2 Glaucoma
TXNRD2 Glucocorticoid Deficiency
TXNRD2 Hearing Loss
TXNRD2 Hypernatriuria
TXNRD2 Hypertrophic cardiomyopathy
TXNRD2 Hypoglycemic coma
TXNRD2 Hypoglycemic seizures
TXNRD2 Lipoatrophy

TXNRD2 Lipodystrophy
TXNRD2 Mental retardation
TXNRD2 Myopathy
TXNRD2 Palmoplantar keratoderma
TXNRD2 Precocious puberty
TXNRD2 Prostatic Neoplasms
TXNRD2 Prostate cancer
TXNRD2 Quadriplegia
TXNRD2 Open Angle Glaucoma
TXNRD2 Testicular adrenal rest tumor
TXNRD1 Adenoma
TXNRD1 Arthritis
TXNRD1 Colorectal Cancer
TXNRD1 Colorectal Neoplasms
TXNRD1 Dermatitis
TXNRD1 Epilepsy
TXNRD1 Mesothelioma

TXNRD1 Myocardial Ischemia
TXNRD1 Osteoarthrosis Deformans
TXNRD1 Papillary Adenoma
PSMB1 Arthritis
PSMB1 Coronary Syndrome
PSMB1 Nervous System Diseases
PSMB1 Osteoarthrosis Deformans
PSMA2 Colorectal Cancer
PSMA2 Heart Diseases
PSMA2 Osteoporosis
PSMB7 Atrial Fibrillation
PSMB7 Paroxysmal atrial fibrillation
PSMB3 Arthritis
PSMB7 Atrial Fibrillation
PSMB7 Paroxysmal atrial fibrillation

PSMA1 Malignant Neoplasm
PSMA1 Cervical Tumor
PSMA1 Stomach Neoplasms
PSMA1 Neoplasms
PSMA1 Nervous System Disorder
PSMA1 Oral Submucous Fibrosis
PSMA1 Cervical Intraepithelial Neoplasia
PSMA1 Head and neck cancer
PSMA1 Cervix carcinoma
PSMA1 Prostate cancer
PSMA1 Stomach Carcinoma
PSMA1 Head And Neck Carcinoma
PSMA1 Cervical Cancer
PSMB4 Acanthosis Nigricans
PSMB4 Anemia
PSMB4 Arthritis
PSMB4 Conjunctivitis

PSMB4 Lipodystrophy
PSMB4 Lymphopenia
PSMB4 Mental Depression
PSMB4 Myocardial Infarction
PSMB4 Myositis
PSMB4 Panniculitis
PSMB4 Sinusitis
PSMB2 Urinary bladder cancer
PSMB2 Bladder Neoplasm
PSMA6 Myocardial Infarction
PSMB9 Acanthosis Nigricans
PSMB9 Anemia
PSMB9 Arthritis
PSMB9 Conjunctivitis
PSMB9 Crohn Disease
PSMB9 Dermatitis
PSMB9 Development Disorder
PSMB9 Diabetes Mellitus

PSMB9 Gianotti-Crosti Syndrome
PSMB9 Lipodystrophy
PSMB9 Lymphopenia
PSMB9 Myositis
PSMB9 Nakajo Syndrome
PSMB9 Narcolepsy
PSMB9 Oral Ulcer
PSMB9 Panniculitis
PSMB9 Proteasome-associated autoinflammatory syndrome
PSMB9 Rheumatoid arthritis
PSMB9 Schizophrenia
PSMB9 Sinusitis
PSMB6 Anorexia
PSMB6 Anxiety Disorder
PSMB6 Rheumatoid arthritis
PSMB6 Asthma
PSMB6 Ataxia Telangiectasia
PSMB6 beta Thalassemia

PSMB6 Blast phase chronic myelogenous leukemia
PSMB6 Burkitt`s Lymphoma
PSMB6 Malignant Neoplasm
PSMB6 Carcinoma
PSMB6 Colonic Neoplasms
PSMB6 Lung carcinoma
PSMB6 Renal Carcinoma
PSMB6 Celiac disease
PSMB6 Congenital Chromosomal Disease
PSMB6 Crohn Disease
PSMB6 Scleroderma
PSMB6 Diabetes Mellitus
PSMB6 Eating Disorders
PSMB6 Guillain-Barre Syndrome
PSMB6 Hemoglobinopathy
PSMB6 Immunologic Deficiency Syndromes

PSMB6 Leukemia
PSMB6 Lymphocytic Leukemia
PSMB6 Lymphoid leukemia
PSMB6 Lymphoblastic Leukemia
PSMB6 Myeloid Leukemia
PSMB6 Myelomonocytic Leukemia
PSMB6 T-cell leukemia
PSMB6 Lupus Erythematosus
PSMB6 Lymphoma
PSMB6 Non-Hodgkin lymphoma
PSMB6 Lymphoproliferative Disorder
PSMB6 Medulloblastoma
PSMB6 Melanoma
PSMB6 Mitral Valve Stenosis
PSMB6 Multiple Sclerosis
PSMB6 Neoplasms

PSMB6 Obesity
PSMB6 Schizophrenia
PSMB6 T-Cell Lymphoma
PSMB6 Follicular Thyroid Carcinoma
PSMB6 Lung Cancer
PSMB6 Miller Dieker Syndrome
PSMB6 Lysinuric Protein Intolerance
PSMB6 Delta-Beta Thalassemia
PSMB6 Delta-Thalassemia
PSMB6 Prostate cancer
PSMB6 Hematologic Neoplasms
PSMB6 Neuropathy
PSMB6 Mood Disorder
PSMB6 Colon Carcinoma
PSMB6 Myeloproliferative disorder
PSMB6 Myelodysplastic Syndrome

PSMB5 Myocardial Ischemia
PSMA5 Fatty Liver
PSMA5 Narcolepsy
PSMA5 Osteoporosis
PSMB10 Atrial Fibrillation
PSMB10 Autoimmune Diseases
PSMB10 Malignant Neoplasm
PSMB10 Cervical Tumor
PSMB10 Crohn Disease
PSMB10 Multiple myeloma
PSMB10 Retinal Diseases
PSMB10 Vitiligo
PSMB10 Hypertensive Retinopathy
PSMB10 Inclusion Body Myositis
PSMB10 Cervix carcinoma
PSMB10 Vitiligo-associated SYSTEMIC LUPUS ERYTHEMATOSUS

PSMB10 Cervical Cancer
PSMB10 Colonic Neoplasms
PSMA3 Proteasome-associated autoinflammatory syndrome
PSMA3 digenic
PSMA4 Anaplastic Carcinoma
PSMA4 Carcinoma
PSMA4 Liver neoplasms
PSMA4 Liver Cancer
PSMA4 Myocardial Infarction
PSMA4 Respiratory Distress Syndrome
PSMA4 Rheumatoid arthritis
PSMB8 Anemia
PSMB8 Arachnodactyly
PSMB8 Arthrogryposis multiplex congenita
PSMB8 Autoinflammatory Disease

PSMB8 Bundle Branch Block
PSMB8 Congestive Heart Failure
PSMB8 Conjunctivitis
PSMB8 Diabetes Mellitus
PSMB8 Elbow flexion contracture
PSMB8 Episcleritis
PSMB8 Erythema nodosum
PSMB8 Glomerulonephritis
PSMB8 Hyperostosis
PSMB8 Kidney Disease
PSMB8 Lipoatrophy
PSMB8 Lipodystrophy
PSMB8 Macroglossia
PSMB8 Macrotia
PSMB8 Mental retardation
PSMB8 Nakajo Syndrome
PSMB8 Osteopenia

PSMB8 Panniculitis
PSMB8 Proteasome-associated autoinflammatory syndrome
PSMB8 Rheumatoid arthritis
PSMA8 Colorectal Cancer
PSMD14 Autoimmune Diseases
PSMD14 Breast Cancer
PSMD14 Malignant Neoplasm
PSMD14 Colorectal Cancer
PSMD14 Esophagus Neoplasm
PSMD14 Multiple myeloma
PSMD14 Neoplasms
PSMD14 Vascular Diseases
PSMD14 Esophageal Carcinoma
PSMD14 Liver Cancer
PSMD14 Prostate cancer
PSMD14 Breast Carcinoma

PSMD14 Attention Deficit Hyperactivity Disorder
PSMD14 Liver carcinoma
PSMD14 Triple Negative Breast Neoplasms
PSMD4 Atrial Fibrillation
PSMD4 Autoimmune Diseases
PSMD4 Breast Cancer
PSMD4 Malignant Neoplasm
PSMD4 Endometrial Cancer
PSMD4 Lung carcinoma
PSMD4 Carcinoma
PSMD4 Cervical Tumor
PSMD4 Colorectal Cancer
PSMD4 Coronary Arteriosclerosis
PSMD4 Coronary Heart Disease
PSMD4 Esophagus Neoplasm
PSMD4 Inflammatory Bowel Disease

PSMD4 Myeloid Leukemia
PSMD4 Lung Neoplasms
PSMD4 Lupus Erythematosus
PSMD4 Stomach Neoplasms
PSMD4 Multiple myeloma
PSMD4 Multiple Sclerosis
PSMD4 Spinal Muscular Atrophy
PSMD4 Myositis
PSMD4 Neoplasms
PSMD4 Neuroblastoma
PSMD4 Papilloma
PSMD4 Stroke
PSMD4 Androgen-Insensitivity Syndrome
PSMD4 Esophageal Carcinoma
PSMD4 Angelman Syndrome
PSMD4 Exudative Macular Degeneration
PSMD4 Cervix carcinoma

PSMD4 Vitelliform Macular Dystrophy
PSMD4 Liver Cancer
PSMD4 Prostate cancer
PSMD4 Endometrial carcinoma
PSMD4 Breast Carcinoma
PSMD4 Stomach Carcinoma
PSMD4 Sarcoma
PSMD4 Pigmentary Disorder
PSMD4 Reticulate
PSMD4 With Systemic Manifestations
PSMD4 Coronary artery disease
PSMD4 Liver carcinoma
PSMD4 Vitreoretinochoroidopathy
PSMD4 Cervical Cancer
PSMD7 Adenocarcinoma
PSMD7 Adenoma
PSMD7 Ameloblastoma

PSMD7 Arteriosclerosis
PSMD7 Arthritis
PSMD7 Rheumatoid arthritis
PSMD7 Asthma
PSMD7 Atherosclerosis
PSMD7 Autoimmune Diseases
PSMD7 Behcet Syndrome
PSMD7 Breast Cancer
PSMD7 Breast Diseases
PSMD7 Malignant Neoplasm
PSMD7 Carcinoma
PSMD7 Ductal carcinoma
PSMD7 Lung carcinoma
PSMD7 Colitis
PSMD7 Ulcerative colitis
PSMD7 Colorectal Cancer
PSMD7 Crohn Disease
PSMD7 Dermatitis
PSMD7 Diabetes

PSMD7 Diabetes Mellitus
PSMD7 Eczema
PSMD7 Graft-Vs-Host Disease
PSMD7 Hamartoma
PSMD7 Hemoglobinopathy
PSMD7 Immunologic Deficiency Syndromes
PSMD7 Inflammatory Bowel Disease
PSMD7 Liver neoplasms
PSMD7 Lung Diseases
PSMD7 Lupus Erythematosus
PSMD7 Non-Hodgkin lymphoma
PSMD7 Multiple Sclerosis
PSMD7 Neoplasms
PSMD7 Peptic Ulcer
PSMD7 Psoriasis
PSMD7 Ankylosing Spondylitis
PSMD7 Giant Cell Arteritis

PSMD7 Lymphoma
PSMD7 Lung adenocarcinoma
PSMD7 Aortic Aneurysm
PSMD7 Neuroendocrine Tumors
PSMD7 Liver Fibrosis
PSMD7 Lung Cancer
PSMD7 Mucosa-associated lymphoma
PSMD7 Psoriasis vulgaris
PSMD7 Esophagus Neoplasm
PSMD7 Impaired Cognition
PSMD7 Prostate cancer
PSMD7 Dementia
PSMD7 Ewing sarcoma
PSMD7 Breast Carcinoma
PSMD7 Pulmonary Fibrosis
PSMD7 Coronary artery disease
PSMD7 Liver carcinoma

SEM1 Aniridia
SEM1 Carcinoma
SEM1 Diverticular Bleeding
SEM1 Diverticular Diseases
SEM1 Ectrodactyly
SEM1 Hearing Loss
SEM1 Isolated Split Hand-Split Foot Malformation
SEM1 Oligodactyly
SEM1 Split hand foot deformity
SEM1 Syndactyly of fingers
ADRM1 Anemia
ADRM1 Rheumatoid arthritis
ADRM1 Breast Cancer
ADRM1 Malignant Neoplasm
ADRM1 Colorectal Cancer
ADRM1 Glioblastoma
ADRM1 Leukemia
ADRM1 Liver Cirrhosis
ADRM1 Stomach Neoplasms

ADRM1 Multiple Sclerosis
ADRM1 Neoplasms
ADRM1 Petechiae
ADRM1 T-Cell Lymphoma
ADRM1 Migraine
ADRM1 Intrahepatic Cholangiocarcinoma
ADRM1 Prostate cancer
ADRM1 Ovarian cancer
ADRM1 Breast Carcinoma
ADRM1 Ovarian neoplasm
ADRM1 Cirrhosis
ADRM1 Liver carcinoma
ADRM1 Oestrogen Receptor Positive Breast Cancer
ADRM1 Ovarian Epithelial carcinoma
PSMD1 Urinary bladder cancer
PSMD1 Bladder Neoplasm
PSMD1 Breast Cancer

PSMD1 Stomach Neoplasms
PSMD1 Breast Carcinoma
PSMD1 Stomach Carcinoma
PSMD1 Bladder carcinoma
PSMD1 Liver carcinoma
PSMD1 Neurodegenerative Disorders
PSMD12 17q24.2 microdeletion syndrome
PSMD12 Cubitus valgus
PSMD12 Anxiety Disorder
PSMD12 Dermatitis
PSMD12 Developmental Delay
PSMD12 Hallucinations
PSMD12 Mental retardation
PSMD12 Micrognathism
PSMD12 Microtia
PSMD12 Mood swings
PSMD12 Motor delay

PSMD12 Myopia
PSMD12 Otitis media
PSMD12 Otosclerosis
PSMD12 Patent ductus arteriosus
PSMD12 Ptosis
PSMD12 Pulmonary Stenosis
PSMD12 Scoliosis
PSMD12 Secondary Physiologic Amenorrhea
PSMD12 STANKIEWICZ-ISIDOR SYNDROME
PSMD12 Syndromic Mental Retardation
PSMD12 Synophrys
PSMD12 Vesicoureteral Reflux
PSMC2 Malignant Neoplasm
PSMC2 Colorectal Cancer
PSMC2 Osteosarcoma
PSMC2 Alveolar Sarcoma
PSMC4 Adenoma

PSMC4 Neoplasms
PSMC4 Parkinson disease
PSMC4 Mammary Neoplasms
PSMC6 Asthma
PSMC6 Carcinoma
PSMC6 Diabetes Mellitus
PSMC6 Glioblastoma
PSMC6 Huntington Disease
PSMC6 Leukemia
PSMC6 Promyelocytic Leukemia
PSMC6 Multiple myeloma
PSMC6 Spinocerebellar Ataxia
PSMC6 Fibroadenoma
PSMC6 Prostate cancer
PSMC6 Brain Infarction
PSMC6 Liver carcinoma
PSMC6 Arthritis
PSMD3 Asthma

PSMD3 Biliary Cirrhosis
PSMC3 Congenital Cataract
PSMD8 Breast Cancer
PSMD8 Malignant Neoplasm
PSMD8 Carcinoma
PSMD8 Lung carcinoma
PSMD8 Diabetes Mellitus
PSMD8 Liposarcoma
PSMD8 Mesothelioma
PSMD8 Neoplasms
PSMD8 Papillary thyroid carcinoma
PSMD8 Lung Cancer
PSMD8 Breast Carcinoma
PSMD8 Mammary Neoplasms
PSMD8 Liver carcinoma
PSMD8 Nasopharyngeal Carcinoma

PSMD8 Neurodegenerative Disorders
PSMC1 Pancreatitis
PSMC1 Ataxia Telangiectasia
PSMC1 Cognitive disorder
PSMC1 Multiple myeloma
PSMC1 Myopia
PSMC1 Parkinson disease
PSMC1 Status Epilepticus
PSMC1 B-Cell Lymphoma
PSMC1 Cervix carcinoma
PSMC1 Impaired Cognition
PSMC1 Neurodegenerative Disorders
PSMD2 Amyotrophic Lateral Sclerosis
PSMD2 Breast Cancer
PSMD2 Malignant Neoplasm
PSMD2 Carcinoma

PSMD2 Cervical Tumor
PSMD2 Senile Dementia
PSMD2 Diabetes Mellitus
PSMD2 Endometriosis
PSMD2 T-Cell Lymphoma/Leukemia
PSMD2 Lymphoma
PSMD2 Machado-Joseph Disease
PSMD2 Stomach Neoplasms
PSMD2 Melanoma
PSMD2 Multiple myeloma
PSMD2 Myopathy
PSMD2 Neoplasms
PSMD2 Nervous System Disorder
PSMD2 Paget disease
PSMD2 Ankylosing Spondylitis
PSMD2 Lung adenocarcinoma
PSMD2 Cervical Intraepithelial Neoplasia

PSMD2 Premature aging
PSMD2 Pica
PSMD2 Lung Cancer
PSMD2 Vesicular Stomatitis
PSMD2 Cervix carcinoma
PSMD2 Malignant lymphoma
PSMD2 lymphocytic
PSMD2 intermediate differentiation
PSMD2 Frontotemporal dementia
PSMD2 B-cell Lymphoma
PSMD2 Dementia
PSMD2 Neurodegenerative Disorders
PSMD2 Skin carcinoma
PSMD2 Breast Carcinoma
PSMD2 Lung carcinoma
PSMD2 Stomach Carcinoma

PSMD2 Lymphoblastic Leukemia
PSMD2 Inclusion Body Myopathy With Paget Disease And Frontotemporal Dementia
PSMD2 Alzheimer disease
PSMD2 Liver carcinoma
PSMD2 Thyroid hemiagenesis
PSMD2 Cervical Cancer
PSMD2 Inclusion Body Myopathy With Paget Disease With Or Without Frontotemporal Dementia
PSMD2 Mantle Cell Lymphoma
PSMD2 Glioblastoma
PSMD13 Autoimmune Diseases
PSMD13 Mental Disorders
PSMD13 Leber Congenital Amaurosis
PSMD13 Mental Depression
PSMD11 Melanoma

PSMD11 Arthritis
PSMD11 Parkinson disease
PSMD11 Ventricular hypertrophy
PSMD11 Pancreatic adenocarcinoma
PSMC5 Colorectal Cancer
PSMC5 Stevens-Johnson Syndrome
PSMD6 Diabetes Mellitus
CA2 Adenocarcinoma
CA2 Anemia
CA2 Autism Spectrum Disorder
CA2 Carcinoma
CA2 Developmental Delay
CA2 Diaphyseal dysplasia
CA2 Distal Renal Tubular Acidosis
CA2 Dwarfism
CA2 Esophagus Neoplasm
CA2 Gastric Cancer
CA2 Liver carcinoma
CA2 Malocclusion

CA2 Mental retardation
CA2 Nervous System Diseases
CA2 Optic Atrophy
CA2 Osteopetrosis
CA2 Osteopetrosis With Renal Tubular Acidosis
CA2 Osteoporosis
CA2 Periodic hypokalemic paresis
CA2 Renal Tubular Acidosis
CA2 Stomach Neoplasms
APP Alzheimer disease
APP Amnesia
APP Cerebral amyloid angiopathy
APP Amyloidosis
APP Anxiety Disorder
APP Aphasia
APP Bipolar Disorder
APP Cardiomyopathy
APP Cerebral cortical atrophy
APP Cerebral Hemorrhage
APP Cognitive disorder

APP Degenerative Diseases
APP Central Nervous System
APP Dementia
APP Developmental Delay
APP Dysgraphia
APP Dysphagia
APP Epileptic encephalopathy
APP Febrile seizures
APP Fragile X Syndrome
APP Hallucinations
APP Impaired Cognition
APP Ischemic Stroke
APP Language Disorders
APP Learning Disorders
APP Leukoencephalopathy
APP Memory Disorders
APP Age-Related Memory Disorders
APP Mental Depression
APP Mental retardation
APP Migraine

APP Myocardial Diseases
APP Neurodegenerative Disorders
APP Oculomotor apraxia
APP Oculovestibuloauditory Syndrome
APP Parkinson Disease
APP Premature aging
APP Semantic Dementia
APP Senile Dementia
APP Senile Paranoid Dementia
APP Senile Plaques
APP Spinal Cord Diseases
APP Stroke
CHRNB4 Chronic Obstructive Pulmonary Disease
CHRNB4 Clonic Seizures
CHRNB4 Coronary artery disease
CHRNB4 Coronary Heart Disease
CHRNB4 Hypotonic seizures

CHRNB4 Jacksonian Seizure
CHRNB4 Lung Neoplasms
CHRNB4 Lung carcinoma
CHRNB4 Lung adenocarcinoma
CHRNB4 Lung Cancer
CHRNB4 Seizure
HSP90AA1 Anaplastic Lymphoma
HSP90AA1 Breast Cancer
HSP90AA1 Mammary Neoplasms
HSP90AA1 Breast Carcinoma
HSP90AA1 Dermatitis
HSP90AA1 Marfan Syndrome
HSP90AA1 Mental Depression
HSP90AA1 Mood Disorder
DHFR Anemia
DHFR Autism
DHFR Breast Cancer
DHFR Mammary Neoplasms

DHFR Breast Carcinoma
DHFR Cerebellar Hypoplasia
DHFR Cerebral Atrophy
DHFR Colorectal Cancer
DHFR Colorectal Neoplasms
DHFR Constitutional Megaloblastic Anemia With Severe Neurologic Disease
DHFR Deficiency Of Dihydrofolate Reductase
DHFR Developmental Delay
DHFR Folic Acid Deficiency
DHFR Inborn Errors Of Metabolism
DHFR Marfan Syndrome
DHFR Megaloblastic anemia
DHFR Miscarriage
DHFR Nervous System Disorder
DHFR Osteosarcoma
DHFR Pancytopenia
DHFR Rheumatoid arthritis
DHFR Subfertility

ADA Adenosine Deaminase Deficiency
ADA Alopecia
ADA Anemia
ADA Asthma
ADA Autism
ADA Autoimmune hemolytic anemia
ADA B-Cell Lymphoma
ADA Aplasia of the thymus
ADA Dermatitis
ADA Eosinophilia
ADA Exfoliative Dermatitis
ADA Hyperemia
ADA Hypothyroidism
ADA Immune Thrombocytopenic Purpura
ADA Lung Neoplasms
ADA Lung Cancer
ADA Lymphoma
ADA Mesangial sclerosis
ADA Multiple gastrointestinal atresias
ADA Nephrotic Syndrome
ADA Omenn Syndrome

ADA Otitis media
ADA Pancreatic adenocarcinoma
ADA Severe combined immunodeficiency disease
ADA Sinusitis
ADA Spinal Cord Diseases
ADA Thyroiditis
PTGER4 Ankylosing Spondylitis
PTGER4 Corpus Luteum Cyst
PTGER4 Endometrioma
PTGER4 Endometriosis
PTGER4 Lymphoma
PTGER4 Ovarian Cysts
SLC9A1 Ataxia-Deafness Syndrome
SLC9A1 Barrett Epithelium
SLC9A1 Barrett esophagus
SLC9A1 Congestive Heart Failure
SLC9A1 Dysarthria

SLC9A1 Heart Failure
SLC9A1 Hypertrophic cardiomyopathy
SLC9A1 Lichtenstein-knorr syndrome
SLC9A1 Motor delay
SLC9A1 Myocardial Infarction
ADORA1 Asthma
ADORA1 Cerebral Infraction
ADORA1 Catalepsy
ADORA1 Cerebral Ischemia
ADORA1 Cerebral Thrombosis
ADORA1 Colorectal Cancer
ADORA1 Myocardial Infarction
ADORA1 Respiratory Tract Diseases
ADORA1 Schizophrenia
ADORA1 Subarachnoid hemorrhage
ADORA1 Transient Ischemic Attack

ACAT2 Cytosolic Acetoacetyl-CoA Thiolase Deficiency
ACAT2 Developmental Delay
HTR3A Autism
HTR3A Bipolar Disorder
HTR3A Mental Depression
HTR3A Mood Disorder
HTR3A Schizophrenia
MGLL Diabetes Mellitus
MGLL Metabolic Syndrome
MGLL Osteoporosis
SRC Urinary bladder cancer
SRC Bladder Neoplasm
SRC Breast Cancer
SRC Mammary Neoplasms
SRC Breast Carcinoma
SRC Carcinoma
SRC Colitis
SRC Colon Cancer
SRC Colon Carcinoma

SRC Colonic Neoplasms
SRC Colorectal Cancer
SRC Colorectal Neoplasms
SRC Grand Mal Status Epilepticus
SRC Hereditary nonpolyposis colorectal cancer
SRC Hereditary Thrombocytopenia With Myelofibrosis
SRC Leiomyosarcoma Of Uterus
SRC Marfan Syndrome
SRC Neoplasms
SRC Nonconvulsive status epilepticus
SRC Petit Mal Status
SRC Platelet-type bleeding disorder
SRC Polycystic kidney disease
SRC Myelofibrosis
SRC Renal Carcinoma
SRC Status Epilepticus
SRC Stomach Neoplasms

SRC Thrombocytopenia
SRC Bladder carcinoma
AGTR1 Allanson Pantzar McLeod Syndrome
AGTR1 Aortic Aneurysm
AGTR1 Pulmonary hypoplasia
AGTR1 Congestive Heart Failure
AGTR1 Diabetic Retinopathy
AGTR1 Essential Hypertension
AGTR1 Heart Failure
AGTR1 Hypertension
AGTR1 Kidney Disease
AGTR1 Microcephaly
AGTR1 Miscarriage
AGTR1 Myocardial Infarction
AGTR1 Narcolepsy
AGTR1 Pancreatic Ductal Carcinoma

AGTR1 Patent ductus arteriosus
AGTR1 Pituitary adenoma
AGTR1 Pituitary Neoplasms
AGTR1 Pituitary carcinoma
AGTR1 Renal Tubular Dysgenesis Of Genetic Origin
AGTR1 Renal Tubular Dysgenesis With Choanal Atresia And Athelia
TPSAB1 Connective Tissue Disease
TPSAB1 Dermatitis
TPSAB1 Dysautonomia
TPSAB1 Irritable Bowel Syndrome
GRIN1 Autism
GRIN1 Bipolar Disorder
GRIN1 Cerebral Atrophy
GRIN1 Developmental Delay
GRIN1 Dyskinetic Syndrome
GRIN1 Dysmorphic Features
GRIN1 Epilepsy

GRIN1 Epileptic encephalopathy
GRIN1 Febrile seizures
GRIN1 Frontal bossing
GRIN1 Hypoplasia Of Corpus Callosum
GRIN1 Mental retardation
GRIN1 Movement Disorders
GRIN1 NEURODEVELOPMENTAL DISORDER WITH HYPERKINETIC MOVEMENTS WITHOUT SEIZURES
GRIN1 Neurodevelopmental Disorder With Or Without Hyperkinetic Movements And Seizures
GRIN1 Non-Syndromic Intellectual Disability
GRIN1 Polymicrogyria
GRIN1 Psychosis
GRIN1 Schizoaffective Disorder
GRIN1 Schizophreniform Disorders
GRIN1 Scoliosis
GRIN1 Strabismus
GRIN1 Trigeminal neuralgia

KDM1A Brachycephaly
KDM1A Camptodactyly of fingers
KDM1A Cleft Palate
KDM1A Psychomotor Retardation
KDM1A And Distinctive Facial Features
KDM1A Colonic Neoplasms
KDM1A Developmental Delay
KDM1A Dwarfism
KDM1A Exotropia
KDM1A Frontal bossing
KDM1A Hypoplasia Of Corpus Callosum
KDM1A Hypoplasia Of Thumb
KDM1A Lung carcinoma
KDM1A Melanoma
KDM1A Motor delay
KDM1A Primary Tethered cord syndrome

KDM1A Prostatic Cancer
KDM1A Castration-Resistant
KDM1A Prostatic Neoplasms
KDM1A Castration-Resistant
KDM1A Syringomyelia
KDM1A T-Cell Lymphoma
SLC5A1 Congenital glucose-galactose malabsorption
SLC5A1 Glucose-Galactose Malabsorption
SLC5A1 Hypertonic dehydration
SLC5A1 Malabsorption Syndrome
GRIN2A Absence Seizure
GRIN2A Agnosia
GRIN2A Anxiety Disorder
GRIN2A Aphasia
GRIN2A Apraxia
GRIN2A Attention Deficit Hyperactivity Disorder
GRIN2A Auditory processing disorder

GRIN2A Autism
GRIN2A Benign Rolandic Epilepsy
GRIN2A Bilateral convulsive seizures
GRIN2A Bipolar Disorder
GRIN2A Colorectal Cancer
GRIN2A Colorectal Neoplasms
GRIN2A Congenital Microcephaly
GRIN2A Continuous spike and waves during slow sleep
GRIN2A Developmental Delay
GRIN2A Dwarfism
GRIN2A Dysarthria
GRIN2A Dyslalia
GRIN2A Dysphasia
GRIN2A Epilepsy
GRIN2A Epilepsy
GRIN2A Focal
GRIN2A With Speech Disorder And With Or Without Mental Retardation

GRIN2A Epileptic Encephalopathy And Intellectual Disability
GRIN2A Febrile seizures
GRIN2A Focal Seizures
GRIN2A Hemifacial seizures
GRIN2A Hypoplasia Of Corpus Callosum
GRIN2A Mental retardation
GRIN2A Landau-Kleffner Syndrome
GRIN2A Language Development Disorders
GRIN2A Laryngospasm
GRIN2A Manic Disorder
GRIN2A Melanoma
GRIN2A Mental Depression
GRIN2A Migraine
GRIN2A Mood Disorder
GRIN2A Mood swings
GRIN2A Rolandic Epilepsy

GRIN2A Rolandic Epilepsy-Speech Dyspraxia Syndrome
GRIN2A Salaam Seizures
GRIN2A Schizophrenia
GRIN2A Seizure
GRIN2A Sleep Apnea
GRIN2A Specific Learning Disorder
GRIN2A Speech Delay
GRIN2A Speech Disorders
GRIN2A Stereotypy
ADORA1 Asthma
ADORA1 Cerebral Infraction
ADORA1 Catalepsy
ADORA1 Cerebral Ischemia
ADORA1 Cerebral Thrombosis
ADORA1 Colorectal Cancer
ADORA1 Myocardial Infarction
ADORA1 Respiratory Tract Diseases

ADORA1 Schizophrenia
ADORA1 Subarachnoid hemorrhage
ADORA1 Transient Ischemic Attack
ADORA3 Colorectal Cancer
ADORA3 Myocardial Infarction
ADORA2A Anxiety Disorder
ADORA2A Cardiomyopathy
ADORA2A Clonic Seizures
ADORA2A Encephalopathy With Biphasic Seizures And Diffusion
ADORA2A Hypotonic seizures
ADORA2A Insomnia
ADORA2A Jacksonian Seizure
ADORA2A Melancholia
ADORA2A Mental Depression
ADORA2A Mood Disorder
ADORA2A Movement Disorders

ADORA2A Myocardial Diseases
ADORA2A Nonorganic Insomnia
ADORA2A Panic Disorder
ADORA2A Psychophysiological Insomnia
ADORA2A Rheumatoid arthritis
ADORA2A Schizophrenia
ADORA2A Seizure
ADORA2A Septicemia
ADORA2A Sleep Disorders
ADORA2A Status marmoratus
SNCA Abnormal Male Sexual Function
SNCA Akinesia
SNCA Anxiety Disorder
SNCA Cerebral cortical atrophy
SNCA Congenital Clubfoot
SNCA Degenerative Diseases
SNCA Central Nervous System
SNCA Delusions

SNCA Dementia
SNCA Dementia Of Frontal Lobe
SNCA Dementia With Lewy Body
SNCA Dysarthria
SNCA Dysautonomia
SNCA Dyskinetic Syndrome
SNCA Dysphagia
SNCA Dyssomnia
SNCA Female sexual dysfunction
SNCA Gastroparesis
SNCA Gaucher Disease
SNCA Hallucinations
SNCA Hepatolenticular Degeneration
SNCA Hereditary Parkinson`s Disease
SNCA Lewy Body Disease
SNCA Male sexual dysfunction
SNCA Mental Depression
SNCA Neurodegenerative Disorders
SNCA Neurogenic Urinary Bladder
SNCA Panic Disorder

SNCA Paranoia
SNCA Parkinson Disease
SNCA Parkinsonian disease
SNCA Parkinsonian-Pyramidal Syndrome
SNCA Ramsay Hunt Paralysis Syndrome
SNCA Restless Legs Syndrome
SNCA Schizophrenia
SNCA Sleep Disorders
SNCA Social Communication Disorder
APP Alzheimer disease
APP Amnesia
APP Cerebral amyloid angiopathy
APP Amyloidosis
APP Anxiety Disorder
APP Aphasia
APP Bipolar Disorder
APP Cardiomyopathy
APP Cerebral cortical atrophy
APP Cerebral Hemorrhage
APP Cognitive disorder

APP Degenerative Diseases
APP Central Nervous System
APP Dementia
APP Developmental Delay
APP Dysgraphia
APP Dysphagia
APP Epileptic encephalopathy
APP Febrile seizures
APP Fragile X Syndrome
APP Hallucinations
APP Impaired Cognition
APP Ischemic Stroke
APP Language Disorders
APP Learning Disorders
APP Leukoencephalopathy
APP Memory Disorders
APP Age-Related Memory Disorders
APP Mental Depression
APP Mental retardation
APP Migraine

APP Myocardial Diseases
APP Neurodegenerative Disorders
APP Oculomotor apraxia
APP Oculovestibuloauditory Syndrome
APP Parkinson Disease
APP Premature aging
APP Semantic Dementia
APP Senile Dementia
APP Senile Paranoid Dementia
APP Senile Plaques
APP Spinal Cord Diseases
APP Stroke
SLCO1B1 Breast Cancer
SLCO1B1 Mammary Neoplasms
SLCO1B1 Breast Carcinoma
SLCO1B1 Carcinoma
SLCO1B1 Chagas Cardiomyopathy

SLCO1B1 Conjugated hyperbilirubinemia
SLCO1B1 Ischemic Stroke
SLCO1B1 Marfan Syndrome
SLCO1B1 Myopathy
SLCO1B1 Rotor Syndrome
MAOB Akinetic-Rigid Variant Of Huntington Disease
MAOB Alzheimer disease
MAOB Anemia
MAOB Autism
MAOB Bipolar Disorder
MAOB Colonic Neoplasms
MAOB Endometrioma
MAOB Endometriosis
MAOB Extra-Adrenal Pheochromocytoma
MAOB Fulminant Hepatic Failure With Cerebral Edema
MAOB Hepatic Coma

MAOB Hepatic Encephalopathy
MAOB Hepatic Stupor
MAOB Huntington Disease
MAOB Mental Depression
MAOB Mood Disorder
MAOB Parkinson disease
MAOB Pheochromocytoma
MAOB Schizophrenia
MAOB Senile Dementia
MAOB Senile Plaques
SNCA Abnormal Male Sexual Function
SNCA Akinesia
SNCA Anxiety Disorder
SNCA Cerebral cortical atrophy
SNCA Congenital Clubfoot
SNCA Degenerative Diseases
SNCA Central Nervous System
SNCA Delusions

SNCA Dementia
SNCA Dementia Of Frontal Lobe
SNCA Dementia With Lewy Body
SNCA Dysarthria
SNCA Dysautonomia
SNCA Dyskinetic Syndrome
SNCA Dysphagia
SNCA Dyssomnia
SNCA Female sexual dysfunction
SNCA Gastroparesis
SNCA Gaucher Disease
SNCA Hallucinations
SNCA Hepatolenticular Degeneration
SNCA Hereditary Parkinson`s Disease
SNCA Lewy Body Disease
SNCA Male sexual dysfunction
SNCA Mental Depression
SNCA Neurodegenerative Disorders
SNCA Neurogenic Urinary Bladder
SNCA Panic Disorder

SNCA Paranoia
SNCA Parkinson Disease
SNCA Parkinsonian disease
SNCA Parkinsonian-Pyramidal Syndrome
SNCA Ramsay Hunt Paralysis Syndrome
SNCA Restless Legs Syndrome
SNCA Schizophrenia
SNCA Sleep Disorders
SNCA Social Communication Disorder
APP Alzheimer disease
APP Amnesia
APP Cerebral amyloid angiopathy
APP Amyloidosis
APP Anxiety Disorder
APP Aphasia
APP Bipolar Disorder
APP Cardiomyopathy
APP Cerebral cortical atrophy
APP Cerebral Hemorrhage
APP Cognitive disorder

APP Degenerative Diseases
APP Central Nervous System
APP Dementia
APP Developmental Delay
APP Dysgraphia
APP Dysphagia
APP Epileptic encephalopathy
APP Febrile seizures
APP Fragile X Syndrome
APP Hallucinations
APP Impaired Cognition
APP Ischemic Stroke
APP Language Disorders
APP Learning Disorders
APP Leukoencephalopathy
APP Memory Disorders
APP Age-Related Memory Disorders
APP Mental Depression
APP Mental retardation
APP Migraine

APP Myocardial Diseases
APP Neurodegenerative Disorders
APP Oculomotor apraxia
APP Oculovestibuloauditory Syndrome
APP Parkinson Disease
APP Premature aging
APP Semantic Dementia
APP Senile Dementia
APP Senile Paranoid Dementia
APP Senile Plaques
APP Spinal Cord Diseases
APP Stroke
SLCO1B1 Breast Cancer
SLCO1B1 Mammary Neoplasms
SLCO1B1 Breast Carcinoma
SLCO1B1 Carcinoma
SLCO1B1 Chagas Cardiomyopathy

SLCO1B1 Conjugated hyperbilirubinemia
SLCO1B1 Ischemic Stroke
SLCO1B1 Marfan Syndrome
SLCO1B1 Myopathy
SLCO1B1 Rotor Syndrome
CHRNA3 Chronic Obstructive Pulmonary Disease
CHRNA3 Clonic Seizures
CHRNA3 Congenital anomalies of kidney and urinary tract
CHRNA3 Coronary Heart Disease
CHRNA3 Development Disorder
CHRNA3 Dysautonomia
CHRNA3 Bronchitis
CHRNA3 Hypotonic seizures
CHRNA3 Jacksonian Seizure
CHRNA3 Lung Neoplasms
CHRNA3 Lung carcinoma

CHRNA3 Lung adenocarcinoma
CHRNA3 Lung Cancer
CHRNA3 Nasopharyngeal Carcinoma
CHRNA3 Schizophrenia
CHRNA3 Seizure
CHRNA4 ACTH Deficiency
CHRNA4 Attention Deficit Hyperactivity Disorder
CHRNA4 Autism
CHRNA4 Clonic Seizures
CHRNA4 Development Disorder
CHRNA4 Epilepsy
CHRNA4 Hypotonic seizures
CHRNA4 Jacksonian Seizure
CHRNA4 Mental Depression
CHRNA4 Minimal Brain Dysfunction
CHRNA4 Nervous System Disorder
CHRNA4 Nocturnal Epilepsy

CHRNA4 Schizophrenia
CHRNA4 Seizure
CHRNA4 Short Sleeper Syndromes
CHRNA4 Sleep Disorders
CHRNA4 Sleep Wake Disorders
IDO1 Anhedonia
IDO1 Breast Cancer
IDO1 Mammary Neoplasms
IDO1 Breast Carcinoma
IDO1 Degenerative Diseases
IDO1 Central Nervous System
IDO1 Endometrioma
IDO1 Endometriosis
IDO1 Marfan Syndrome
IDO1 Mental Depression
IDO1 Neurodegenerative Disorders
IDO1 Obesity
SCN3A Arthritis
SCN3A Attention Deficit Hyperactivity Disorder

SCN3A Autism
SCN3A Cerebral Atrophy
SCN3A Cryptogenic Partial Complex Epilepsy
SCN3A Developmental Delay
SCN3A Developmental regression
SCN3A Dwarfism
SCN3A Dysautonomia
SCN3A Dyskinetic Syndrome
SCN3A Dysphagia
SCN3A Epilepsy
SCN3A Epileptic encephalopathy
SCN3A Gastroesophageal Reflux Disease
SCN3A Hypodontia
SCN3A Hypoplasia Of Corpus Callosum
SCN3A Mental retardation
SCN3A Microcephaly

SCN3A Non-Specifi Epileptic Encephalopathy
SCN3A Nystagmus
SCN3A Optic Atrophy
SCN3A Polymicrogyria
SCN3A Ptosis
SCN3A Spastic Quadriplegia
SCN3A Status Epilepticus
FNTB Multiple Sclerosis
CHRM1 Cerebral Ischemia
CHRM1 Clonic Seizures
CHRM1 Epilepsy
CHRM1 Hypotonic seizures
CHRM1 Jacksonian Seizure
CHRM1 Memory Disorders
CHRM1 Age-Related Memory Disorders
CHRM1 Schizophrenia
CHRM1 Seizure

PTPN2 Ankylosing Spondylitis
PTPN2 Apraxia
PTPN2 Arthritis
PTPN2 Asthma
PTPN2 Autoimmune Diseases
PTPN2 Carcinoma
PTPN2 Celiac disease
PTPN2 Cholangitis
PTPN2 Crohn Disease
PTPN2 Crohn`s Disease Of Large Bowel
PTPN2 Crohn`s Disease Of The Ileum
PTPN2 Diabetes Mellitus
PTPN2 Esophagus Neoplasm
PTPN2 Ileocolitis
PTPN2 Immune System Diseases
PTPN2 Immunoglobulin A deficiency
PTPN2 Inflammatory Bowel Disease
PTPN2 Iridocyclitis
PTPN2 Lymphoblastic Leukemia
PTPN2 Multiple Sclerosis

PTPN2 Oligoarticular Arthritis	GRIN2B Autism Spectrum Disorder	GRIN2B Mental retardation
PTPN2 Pauciarticular chronic arthritis	GRIN2B Autism	GRIN2B Leukemia
PTPN2 Polyarthritis	GRIN2B Bipolar Disorder	GRIN2B Macrotia
PTPN2 Seronegative polyarthritis	GRIN2B Congenital Epicanthus	GRIN2B Mental Depression
PTPN2 Psoriasis	GRIN2B Craniosynostosis	GRIN2B Neurodevelopmental Disorders
PTPN2 Rheumatoid arthritis	GRIN2B Developmental Delay	GRIN2B Non-Syndromic Intellectual Disability
PTPN2 Rheumatoid Factor-Negative Polyarticular Arthritis	GRIN2B Developmental regression	GRIN2B Schizophrenia
PTPN2 Selective Immunoglobulin A Deficiency	GRIN2B Dyskinetic Syndrome	GRIN2B Scoliosis
PTPN2 Still Disease	GRIN2B Dysphagia	GRIN2B Spasms Syndrome
PTPN2 T-cell leukemia	GRIN2B Epilepsy	GRIN2B Synophrys
PTPN2 Ulcerative colitis	GRIN2B Epileptic encephalopathy	GRIN2B West Syndrome
GRIN2B Astigmatism	GRIN2B Impaired Cognition	

Table 04: The Gene and Disease as found in integrated network analysis was shown here in tabular forms.

This study proposes that protein kinase C (PKC) could serve as a crucial target for the bioactive compounds present in *Zingiber rubens*, a plant known for its diverse phytochemical profile. By exploring the interactions between these phytoconstituents and PKC, we aim to better understand their potential therapeutic effects and the mechanisms through which they may exert their influence.

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EXPERIMENTAL ANIMAL MODEL:

In this study, we utilized adult male Wistar albino rats, each weighing between 160 and 180 grams, which were kindly supplied by M/S Chakraborty Enterprise located in Kolkata (Registration No. 1443/PO/Bt/s/11/CPCSEA). Prior to the commencement of the experimental procedures, these rats exhibited fasting blood glucose (FBG) levels ranging from 85 to 90 mg/dL. To ensure that the animals were well-adjusted to their new environment, we provided a comprehensive acclimatization period of 7 days during which they had unlimited access to both food and fresh water [1].

The housing conditions for the rats were meticulously controlled; the temperature in the facility was maintained at a comfortable 25°C, complemented by a consistent 12-hour light/dark cycle to simulate natural conditions. During the study, the rats were fed a standard high-fat diet, which was designed to meet their nutritional needs while also facilitating our research objectives [2-3].

All protocols related to the experimentation on these animals were rigorously reviewed and received the necessary approvals from the Animal Ethical Committee at Jadavpur University, situated in Kolkata, West Bengal, India. The approved research protocol was granted the official approval number JU/IAEC-22/47 on June 15th, 2023, ensuring adherence to ethical standards in animal research [2].

Oral glucose tolerance test (OGTT)

The study was conducted on rats that had been deprived of food overnight, ensuring that they maintained normal blood sugar levels, specifically ranging between 85-90 mg/dl [1-4]. A total of eighteen rats were divided into three distinct groups, with each group consisting of six rats. The rats in Groups II and III were administered a herbal extract, referred to as SHME, at two different doses of 200 mg/kg and 400 mg/kg body weight, respectively. In contrast, the rats in Group I received only distilled water at a dosage of 5 mL/kg body weight, serving as the control group for this experiment [1-5].

Following the treatment, all rats were given an oral glucose solution at a dosage of 2 g/kg body weight, which was intended to assess their glucose metabolism. Blood samples were meticulously collected from the tail vein at specified intervals—just prior to glucose administration and then at 30, 60, 120, and 240 minutes post-administration. Blood glucose levels were measured using an Accu-Chek glucometer, which provided precise readings to

analyze the effects of the SHME on glucose absorption and processing. This research was carried out in accordance with the methodologies established by Jana et al. in 2023 [1].

Animal model

The potential toxicity of HAZR was thoroughly investigated through a detailed acute oral toxicity study, adhering closely to the guidelines established by the Organisation for Economic Co-operation and Development (OECD) in 2008, specifically the OECD 425 protocols. The study design included a 28-day treatment period during which the subjects were placed on a high-fat diet, followed by a subsequent 14-day observation phase to monitor any adverse effects. Thirty rats were utilized in the experiment, with six individuals assigned to each of the five distinct treatment groups, ensuring a well-rounded assessment of HAZR's safety profile [1-4].

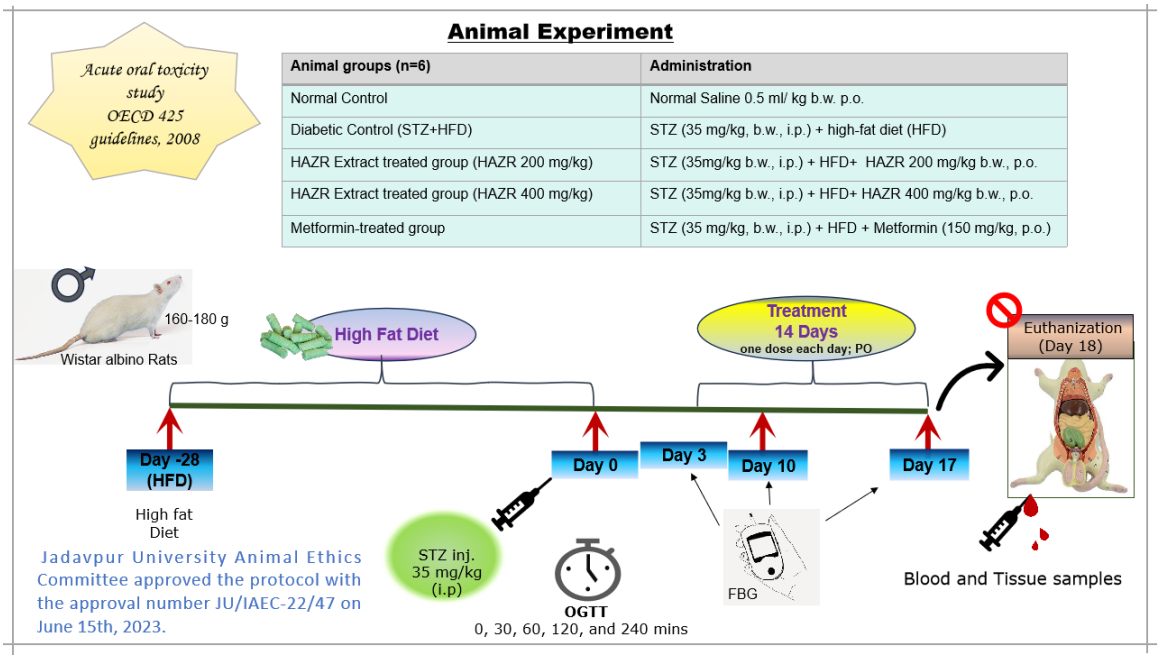

Figure 1: Animal Experiment plan.

- ✓ **Group –I**: Normal control (NC) group: The rats received -normal saline (0.5 ml/kg, p.o.) for 14 days
- ✓ **Group-II**: Diabetic (STZ+HFD) control group: Diabetic group consisted of rats treated with STZ (35 mg/kg, b.w., i.p.) and high-fat diet.
- ✓ **Group-III:** HAZR Extract (SHME 200 mg/kg) treated group: The diabetic rats were treated orally with HAZR (200mg/kg b.w.) for 14 days.
- ✓ **Group-IV:** HAZR Extract (SHME 400 mg/kg) treated group: The diabetic rats were orally treated with HAZR (400mg/kg b.w.) for 14 days.

- ✓ **Group-V:** Metformin (MET)-treated group: The diabetic rats were treated with metformin (150 mg/kg, p.o.) for 14 days.

The next day after the last dose of HAZR, all animals were sacrificed, and the required blood and tissue samples were collected for further analysis [1-4].

Estimation of glycosylated haemoglobin level (HbA1c)

The glycosylated hemoglobin levels were assessed using a commercially available test kit from Coral Clinical System, manufactured by Tulip Diagnostics Pvt. Ltd. in India. A complete blood sample was analyzed for this evaluation, employing a sophisticated assay methodology incorporating ion exchange resin. This approach enhances the accuracy of the measurement, allowing for a precise determination of glycosylated hemoglobin levels, which are crucial for monitoring long-term blood sugar control.

Estimation of serum biochemical parameters

The serum samples collected for analysis underwent thorough examination using a specialized assay kit sourced from Arkray Healthcare Pvt. Ltd. in India. This comprehensive assay evaluated a range of critical biochemical factors, including total protein levels, serum alkaline phosphatase (SALP), serum glutamic pyruvic transaminase (SGPT), and serum glutamic oxaloacetic transaminase (SGOT). Each of these factors plays a crucial role in assessing liver function and overall metabolic health, providing valuable insights into the physiological state of the samples analyzed. [1-4].

Serum lipid profile

We conducted an analysis of the serum lipid profiles in rats induced with diabetes via a high-fat diet and streptozotocin (HFD-STZ). To achieve this, we utilized commercially available assay kits provided by Arkray Healthcare Pvt. Ltd., a reputable supplier based in India. Our examination focused on key lipid metrics, including total cholesterol levels, HDL (high-density lipoprotein) cholesterol, and triglycerides, to better understand the metabolic changes associated with diabetes in this model. [1-4].

Histopathological studies

In the process of conducting histopathological investigations, researchers obtained pancreatic tissue samples from rats that had been humanely sacrificed. The collected tissue was carefully washed with normal saline to remove any impurities. It was then immersed

in 10% formalin, where it remained for a minimum of 24 hours to ensure proper fixation and preservation of cellular structures. After fixation, the tissue underwent dehydration in a series of alcohol solutions to remove water content, making it suitable for embedding. Subsequently, the dehydrated tissue was embedded in paraffin wax, which provided a solid medium for cutting. The resultant blocks were then sliced into thin sections measuring 4 to 5 micrometers in thickness. Finally, these sections were stained using hematoxylin and eosin dye, a standard technique that highlights different cell types and structures, facilitating thorough microscopic examination [1].

Statistical analysis

All results in this study are presented as the mean along with the standard error of the mean (SEM) to provide a clear understanding of the data variability. For the statistical analysis, we utilized GraphPad Prism version 8.02 software. We conducted a one-way analysis of variance (ANOVA) to compare the means of different groups and subsequently applied the post hoc Dunnett test. This method allowed us to assess the statistical significance of our findings. A *p*-value of less than 0.05 was established as the threshold for determining significance, indicating that any observed differences were unlikely to have occurred by chance [1,4].

RESULTS:

Oral glucose tolerance test (OGTT)

Rats exhibiting normal blood sugar levels, typically ranging from 85 to 90 mg/dl, were subjected to an oral blood glucose tolerance test to assess their metabolic response. After the administration of glucose, blood glucose levels initially rose sharply over the first 30 minutes, indicating a robust response to the glucose challenge. Following this peak, there was a gradual decline in blood glucose concentrations over the subsequent 60, 90, and 120 minutes, reflecting the body's mechanisms for glucose regulation, as detailed in Table 1. Notably, the administration of HAZR at a dosage of 400 mg/kg body weight resulted in a significant reduction in blood glucose levels in the tested animals during the oral glucose tolerance test (OGTT), suggesting a potential therapeutic effect of HAZR on glucose metabolism.

Animal model

An acute oral toxicity study of HAZR was conducted in accordance with the OECD 425 guidelines established in 2008. The results revealed no signs of toxicity following the administration of HAZR at doses up to 2000 mg/kg body weight. For this particular investigation, two specific doses of HAZR were carefully selected for evaluation: a low dose of 200 mg/kg body weight and a higher dose of 400 mg/kg body weight. These doses were chosen to thoroughly assess the potential effects of HAZR at varying levels of exposure.

Groups	0 Min	30 Min	60 Min	120 Min
Normal control	89.83±3.9	125.7±2.9	115.7±1.5	110.0±2.08
LD- HAZR (200)	90.33±1.2	126.0±1.5	120.3±1.8	105.7±1.4
HD- HAZR (400)	85±1.5	120.3±1.2*	111.3±2.4*	96.6±2.02*
Metformin 150 mg/kg	81.5±2.5	108±2.30*	98.2±3.20*	87±1.80*

Table 01:Effect of HAZR on oral glucose tolerance test. Values are expressed as mean ±SEM (n=6). *Values significantly differ from normal control where p < 0.05.

Effect on glycosylated haemoglobin level (HbA1c)

In a study involving low-dose STZ high-fat-induced diabetic rats, it was observed that these diabetic animals had markedly elevated fasting blood glucose (FBG) levels when compared to the normal control group, with a statistically significant difference ($p<0.05$). To investigate the effects of HAZR, the diabetic rats were administered this compound for a duration of 14 days at two different doses: 200 mg/kg and 400 mg/kg. Remarkably, treatment with HAZR resulted in a noteworthy reduction in FBG levels, bringing them closer to normal values when assessed against the diabetic control group, again showing

statistical significance ($p<0.05$). The specific changes in fasting blood glucose levels (measured in mg/dL) following HAZR treatment are detailed in Table 2. Furthermore, the rats treated with HAZR displayed a significant decrease in glycosylated hemoglobin levels in comparison to their diabetic counterparts, which was also statistically significant ($p<0.05$), as depicted in Figure 2. This suggests that HAZR may have favorable effects in managing glucose levels in diabetic conditions.

Groups	**Diabetic Animal (After 28th Days of HFD and Single dose STZ treatment)**		
	Day 0	Day 7	Day 14
Normal control	76±12.23	81.33±11.80	88.66±13.41
STZ (45 mg/kg)	410.33±13.52a*	418.66±12.19a*	515±11.15a*
STZ + LD	422.63±12.13	397.33±11.75b*	338.41±12.04b*
STZ + HD	409.33±12.10	306.34±11.45b*	216.33±11.46b*
STZ + metformin (150 mg/kg)	405.31±13.33	259.22±12.10b*	108.66±11.26b*

Figure 02: Effect of HAZR on fasting blood glucose (mg/dL). Values are expressed as mean ±SEM (n=6). *Values significantly differ from normal control where $p < 0.05$.

Estimation of serum biochemical parameters

In comparison to the control group subjected to a low-dose STZ high-fat diet, the groups treated with HAZR showed a remarkable reduction ($p<0.05$) in key blood biochemical markers, specifically SALP, SGOT, and SGPT. This indicates a potential improvement in liver function. Furthermore, the treated groups displayed an increase in total protein levels

compared to their control counterparts, highlighting the beneficial effects of HAZR on overall protein synthesis (refer to Fig. 4). These serum biochemical findings strongly suggest that HAZR possesses hepatoprotective properties, demonstrating its effectiveness at both low and high doses of the plant extract in promoting liver health.

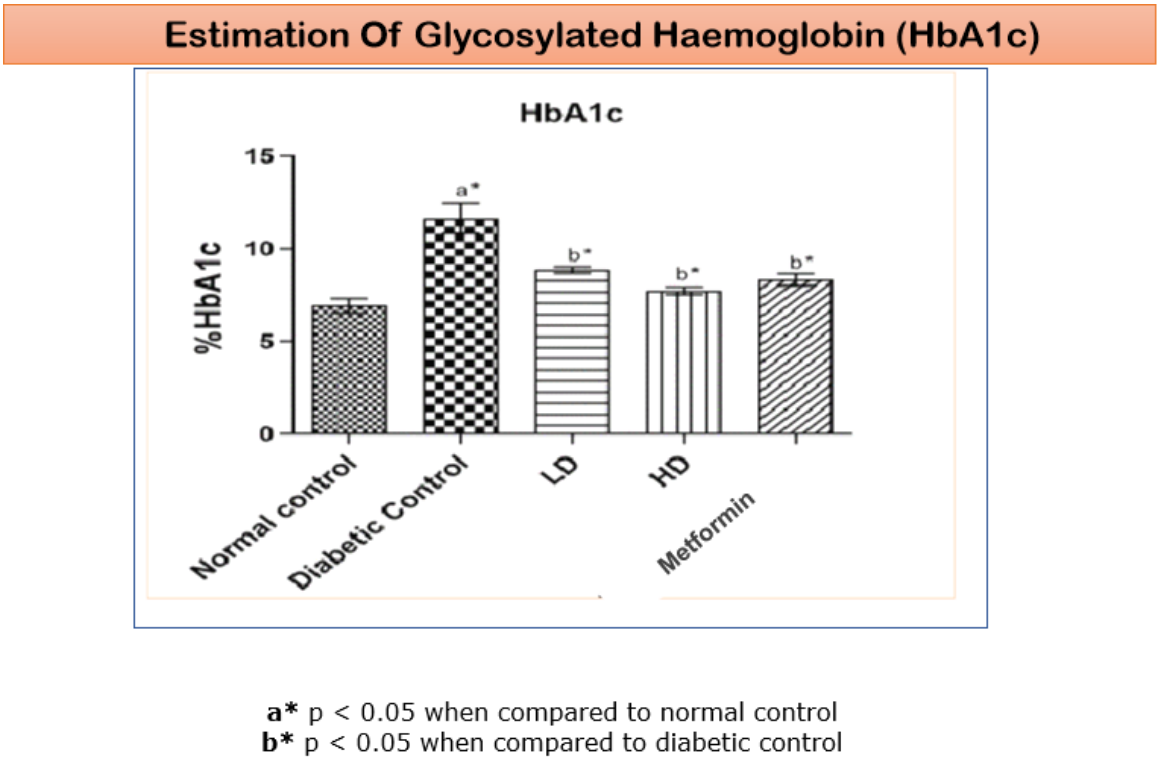

Figure 02: Estimation of Glycosylated Haemoglobin (HbA1c)

Serum lipid profile

The serum lipid profiles of diabetic rats revealed marked alterations when compared to their normal counterparts. Specifically, the diabetic rats exhibited significantly elevated levels of triglycerides and total cholesterol, with statistical significance indicated at $p < 0.05$. In contrast, the levels of HDL (high-density lipoprotein) were notably reduced in these diabetic subjects, also reaching a significant level at $p < 0.05$.

Following the administration of HAZR extract at doses of 200 mg/kg and 400 mg/kg, a remarkable change was observed in the lipid profiles of the diabetic rats. Both triglyceride and total cholesterol levels experienced substantial reductions, again with a significance level of $p < 0.05$. Moreover, the intervention led to a noteworthy increase in HDL levels, emphasizing the potential lipid-regulating effects of HAZR extract in diabetic conditions (refer to Fig. 5 for visual representation).

Histopathological studies

In a comprehensive study comparing HAZR-treated rats to diabetic rats, several significant biochemical changes were observed. The level of malondialdehyde (MDA), a marker for oxidative stress, was notably reduced in the HAZR-treated group ($p<0.05$), indicating a potential protective effect against cellular damage. In contrast, the levels of superoxide dismutase (SOD) and glutathione (GSH), both essential antioxidants that help combat oxidative stress, were significantly elevated ($p<0.05$) in the same group. This suggests that HAZR treatment not only reduces harmful oxidative stress but also enhances the body's own antioxidant defenses. Furthermore, the levels of malondialdehyde were consistently lower ($p<0.05$), as illustrated in Figure 5.

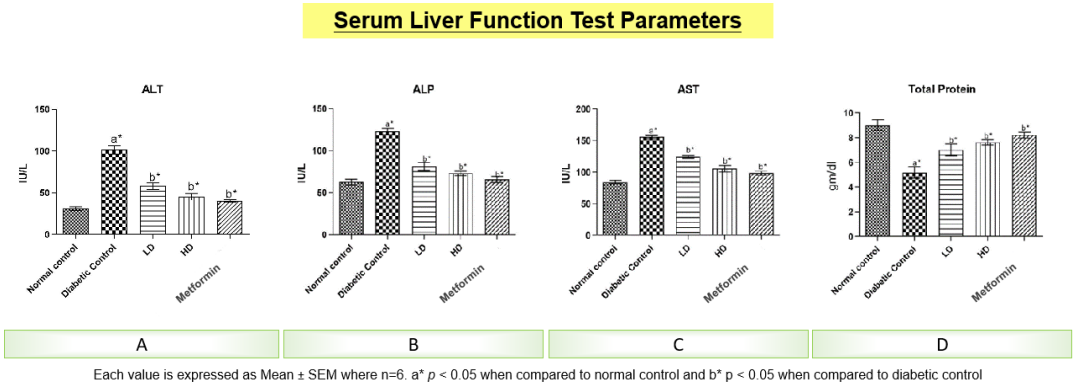

Figure 03: Serum Liver Function Test Parameters. Each value is expressed as Mean ± SEM where n=6. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to diabetic control

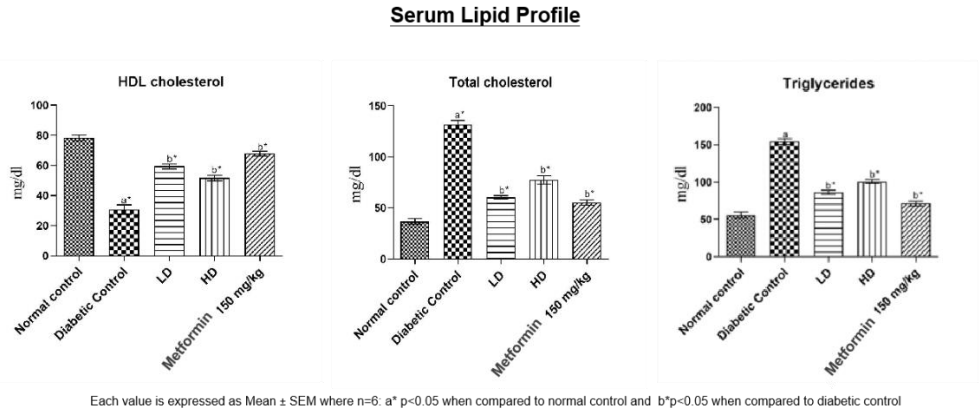

Figure 04: Serum Lipid Profile. Each value is expressed as Mean ± SEM where n=6: a* p<0.05 when compared to normal control and b*p<0.05 when compared to diabetic control.

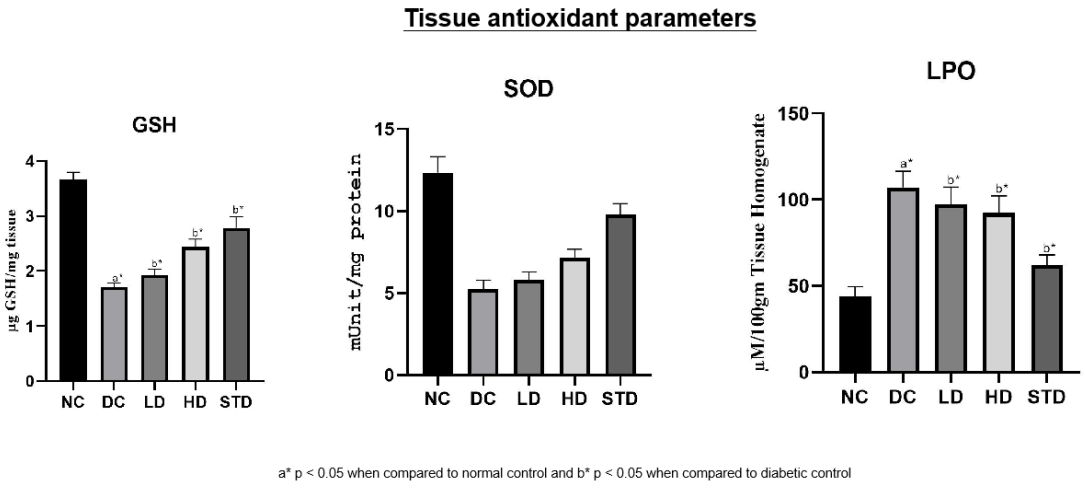

Figure 05: Tissue antioxidant parameters. a* p < 0.05 when compared to normal control and b* p < 0.05 when compared to diabetic control

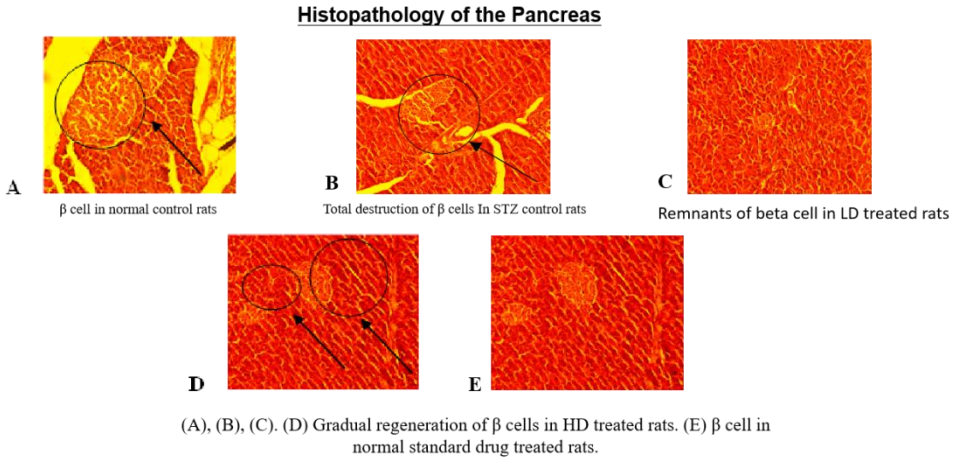

Figure 06: Histopathology of the Pancreas.

Moreover, histopathological examinations of pancreatic tissue revealed compelling evidence of HAZR's protective effects on pancreatic beta cells, which are crucial for insulin production. Remarkably, when comparing the treated groups to diabetic rats, there was a clear, dose-dependent improvement in the density of these vital beta cells. This gradual enhancement underscores the beneficial impact of HAZR treatment on pancreatic health, as depicted in Figure 6. Overall, these findings highlight the potential of HAZR to mitigate the adverse effects of diabetes on pancreatic structure and function.

The objective of this study was to delve into the longstanding traditional belief regarding the antidiabetic properties of the Zingiber rubens Roxb. plant. We employed an animal model specifically designed to mimic non-insulin-dependent diabetes mellitus (NIDDM), more commonly referred to as type 2 diabetes mellitus in humans. This model was established by inducing diabetes in rats through a combination of a high-fat diet (HFD) and low doses of streptozotocin (STZ) [1, 4]. Our findings indicated that this dietary regimen, paired with STZ, led to a significant elevation in blood glucose levels among the experimental subjects. However, a noteworthy observation was made regarding the dose-dependent anti-diabetic effects of HAZR, as elaborated in the results section [8-15].

It is important to recognize that diet plays a pivotal role in the onset and progression of various health conditions, including diabetes, hypertension, hyperlipidaemia, and nephropathy, which can develop over time. Streptozotocin exerts its effects by penetrating the pancreatic β cells via a glucose transporter known as GLUT2. Once inside, it triggers a damaging process that alkylates DNA, leading to oxidative stress and subsequent harm to these insulin-producing cells [17-18]. Consequently, this combination of a high-fat diet and low-dose STZ to induce diabetes in rats has become a widely accepted model for evaluating potential antidiabetic agents.

Zingiber rubens Roxb. is a plant that thrives abundantly in the northeastern region of India, celebrated for its diverse range of biological properties, including its antioxidant, antidiabetic, and immunomodulatory effects [6-8]. The root oil extracted from Z. rubens comprises an impressive variety of 24 chemical compounds, with monoterpenes dominating the composition at 75.3%. Among these, (Z)-citral stands out at 30.1%, followed by camphene at 9.7%, β-phellandrene at 7.5%, and 1,8-cineole at 7.0%. The oil also contains a notable concentration of sesquiterpenoids, with zingiberene being the most prominent at 5.3% [8]. Additionally, the leaves of this plant are rich in various bioactive constituents, including reducing sugars, anthraquinones, terpenoids, flavonoids, saponins, tannins, alkaloids, and cardiac glycosides. An initial study suggested that the high levels of flavonoids and phenolic compounds present in Zingiber rubens may underlie its potent free radical scavenging capabilities [8-32]. To further investigate this property, we conducted an in vitro free radical scavenging assay, yielding significant IC50 values, which are detailed in the results section.

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PROTEIN KINASE C:

Protein kinase C (PKC) is an important family of enzymes classified as serine/threonine kinases, and it is expressed throughout various tissues in the body. These enzymes exist in multiple isoforms, each produced by the transcription of distinct genes, allowing for specialized distribution and function in different tissue types. The isoforms of PKC can be categorized based on their ability to interact with two critical regulatory molecules: calcium ions (Ca2+) and diacylglycerol (DAG), both of which enhance the activity of these kinases [1-2].

The conventional PKC isoforms — specifically α, β1, β2, and γ — are characterized by their ability to bind to both calcium and diacylglycerol, which are essential for their activation. In contrast, the novel PKC isoforms, including δ, ε, η, and θ, interact with diacylglycerol but do not bind calcium, altering their activation dynamics. Meanwhile, the atypical PKC isoforms, such as ζ and ι, are unique in that they do not bind either of these activators [1].

Recent research has revealed that PKC plays a significant role in insulin signal transduction, contributing to our understanding of the mechanisms behind insulin resistance. In particular, studies conducted on C2C12 myocytes—a type of muscle cell—have demonstrated that the stimulation of these cells with phorbol ester, a potent PKC activator, results in the phosphorylation of insulin receptor substrate 1 (IRS-1) at serine residue 1101. This phosphorylation occurs through a pathway dependent on PKC θ and is noteworthy for not involving the common downstream signaling protein, Akt, thus highlighting the unique regulatory pathways influenced by different PKC isoforms in metabolic processes [2-6].

Cells and PKC inhibition Assay by ELISA [1-3]:

Mouse Peritoneal Macrophages, sourced from Cellbiologics (Catalog No. 5032TF), were cultured in RPMI medium enriched with 10% Fetal Bovine Serum (FBS) under a controlled environment of 5% carbon dioxide. Within this optimal growth setting, over 95% of the viable cells were subjected to treatment with lipopolysaccharide (LPS) at a concentration of 25 µg/ml, alongside various concentrations of HAZR ranging from 0 to 100 µg/ml. This experimentation was conducted in 96-well plates to ensure uniformity. To assess the levels of Protein Kinase C (PKC) and phosphorylated PKC (Phospho-PKC), commercially

available Sandwich ELISA kits were utilized (KNH11806 for PKC and KLM0591 for Phospho-PKC).

The process of cell culture begins with the careful seeding of cells in a growth medium specifically designed to provide essential nutrients and support, tailored to the unique requirements of the particular cell type being cultured [Segeritz & Vallier, 2017]. For the cultivation of peritoneal macrophages, Dulbecco's Modified Eagle Medium (DMEM) is the preferred choice.

In this procedure, peritoneal macrophages are gently introduced into a T25 flask containing 3 ml of DMEM. This medium is enriched with 10% fetal bovine serum (FBS) to promote cell growth and viability, along with 1% of antifungal and antibacterial agents to prevent contamination. Additionally, 2.5 g/l of sodium bicarbonate is incorporated into the medium to help stabilize the pH, thus creating an optimal environment for cell growth while ensuring minimal stress during the process.

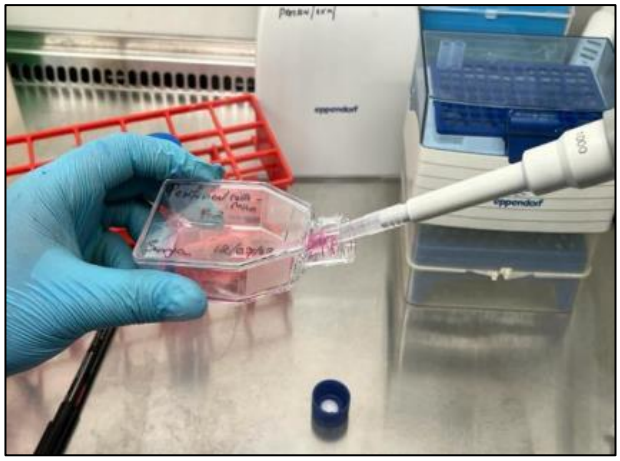

Figure 1: Cell seeding.

The flasks are then placed in an incubator set to 37 °C and maintained with an atmosphere of 5% CO2 to mimic physiological conditions. Throughout the culture period, careful attention is paid to the cells' adherence, morphology, and any signs of contamination. Media is refreshed every 2 to 3 days, and cells are passaged as necessary to promote continued growth and productivity. Observations of the cells are conducted using a fluorescent microscope, utilizing the Trans channel at magnifications of either 10X or 20X to assess the health and characteristics of the cultured macrophages.

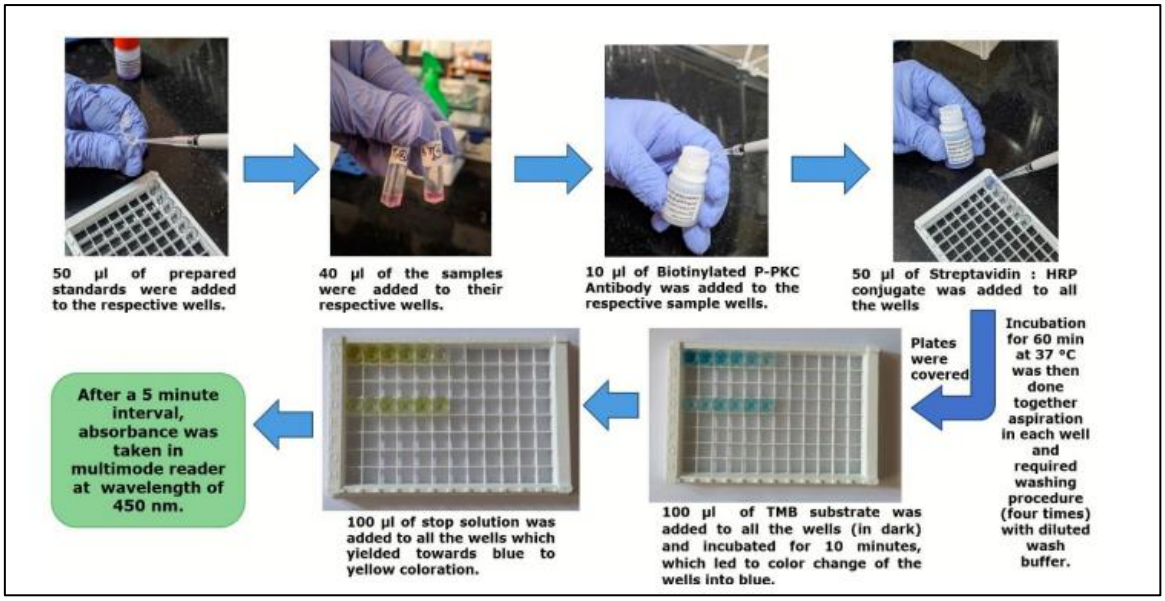

Figure 02: The procedure of the ELISA test for PKC and pPKC.

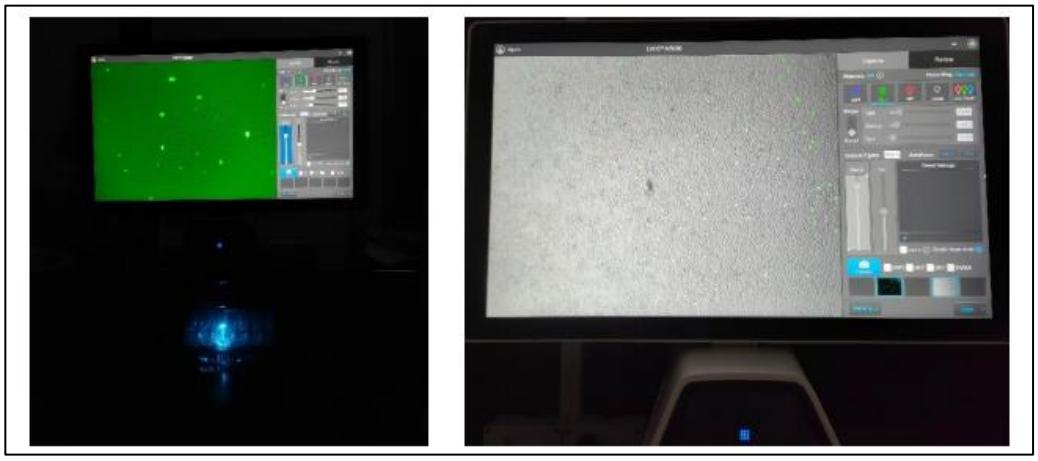

Figure 03: The Automated cell counter.

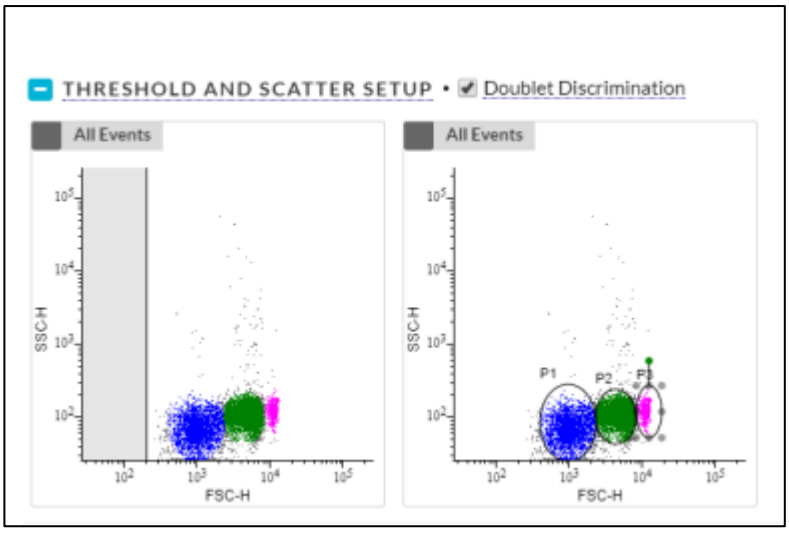

Figure 04: The cell distribution id FACS .

Results:

In our investigation of the molecular mechanisms underlying the action of HAZR, we focused on its effect on the phosphorylation of protein kinase C (PKC) in mouse peritoneal macrophage cells. PKC plays a crucial role in the phosphorylation process of AMP-activated protein kinase (AMPK), which is vital for endocrine regulation. Our findings revealed that HAZR significantly suppresses the phosphorylation of PKC, particularly in response to stimulation with lipopolysaccharide (LPS). This is clearly illustrated by the observed gradual increase in PKC levels at various escalating concentrations of the plant extract. Conversely, we noted a decrease in the levels of phosphorylated PKC (p-PKC), which highlights the inhibitory action of HAZR on the phosphorylation of PKC. These results are depicted in detail in Figure 5 (A-D).

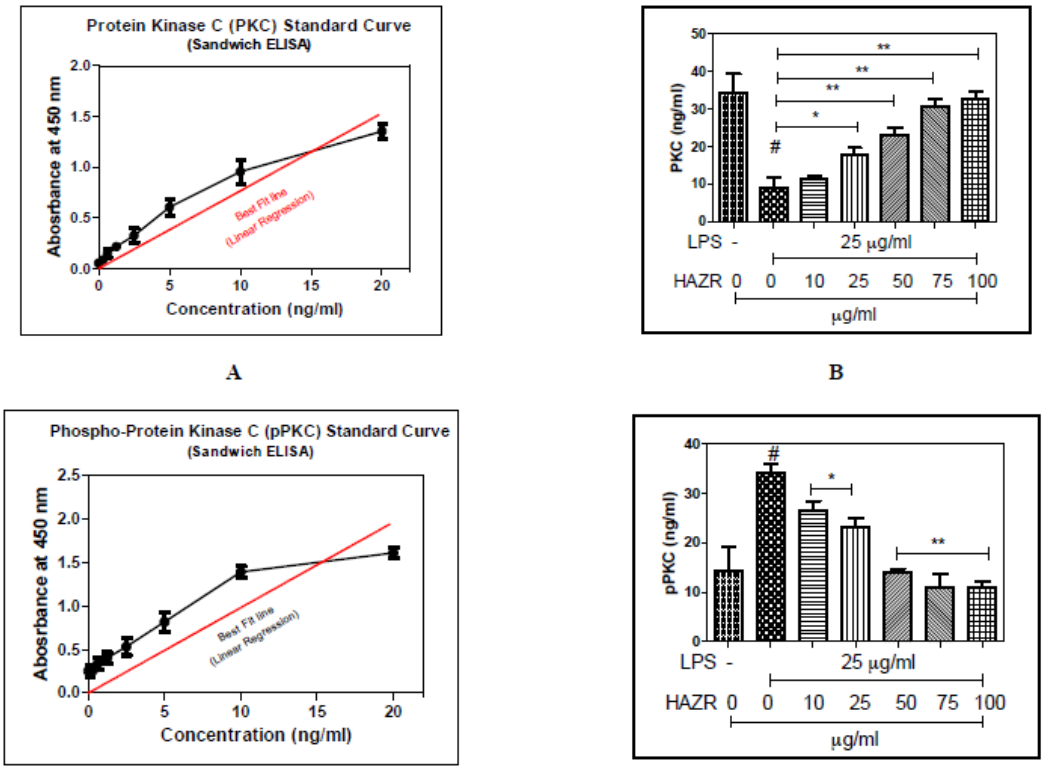

Figure 05: PKC and Phospho- PKC estimation of mouse peritoneal macrophages. Primary macrophages were cultured in RPMI media with 10% FBS in control condition in 5% CO2 incubator. Treated with or without LPS and different concentration of HAZR followed by estimation of PKC and Phospho- PKC estimation by using commercially available Sandwich ELISA kit. All values are expressed as Mean±SEM (n=3). (A) Standard Curve of PKC. (B) PKC levels in cells treated with HAZR and LPS. (C) Standard Curve of Phospho- PKC. (D) Phospho- PKC levels

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The primary objective of this study was to delve into the traditional belief surrounding the Zingiber rubens Roxb. plant, particularly its reputed antidiabetic properties. To explore this hypothesis, we employed an animal model representing non-insulin-dependent diabetes mellitus (NIDDM), commonly known as type 2 diabetes mellitus in humans. This was achieved by inducing diabetes in laboratory rats through a combination of a high-fat diet (HFD) and low doses of streptozotocin (STZ) [1, 4].

Our findings revealed that the combination of HFD and a minimal dose of STZ led to a significant rise in blood glucose levels among the experimental subjects. Notably, we observed a dose-dependent anti-diabetic effect of the Zingiber rubens extract (HAZR), which is detailed in the results section. Existing research has long established that dietary habits play a crucial role in the progression of diabetes, hypertension, hyperlipidaemia, and nephropathy over time.

Streptozotocin (STZ), a compound known for its selective toxic effects on pancreatic β cells, enters these cells via the glucose transporter known as GLUT2. Once inside, it triggers a process that leads to the alkylation of DNA, resulting in damage mediated by oxidative stress. This mechanism underscores why the HFD combined with low-dose STZ-treated animal model is extensively utilized for assessing potential antidiabetic compounds and lead molecules in scientific research.

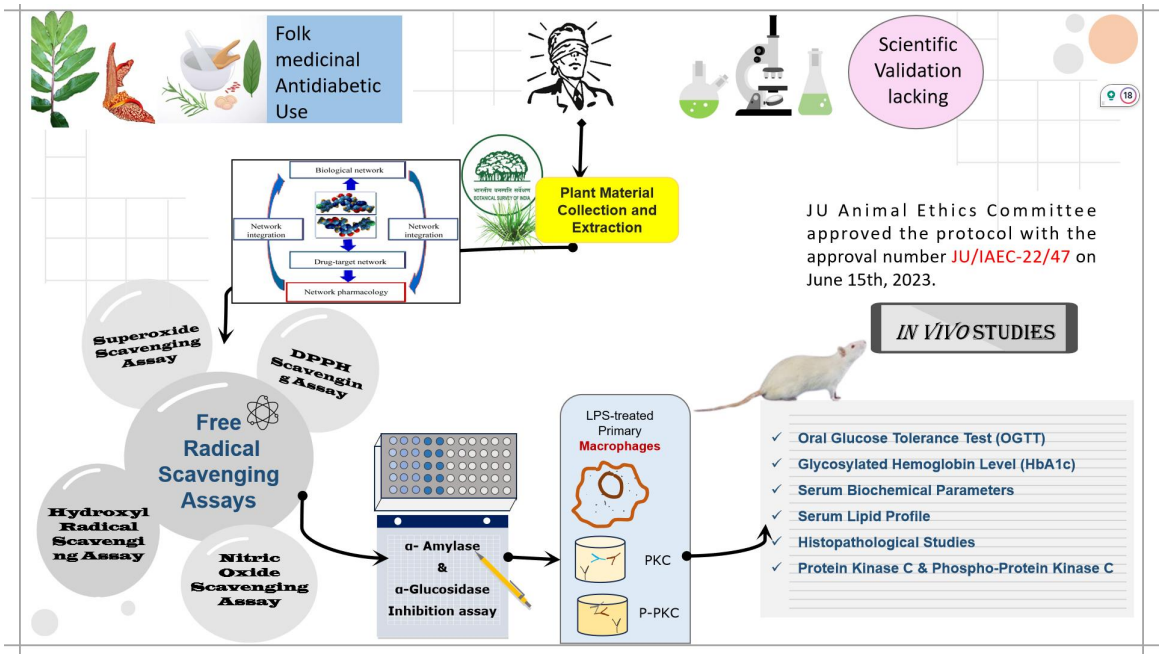

Figure 01: Schematic representation of the whole work.

Zingiber rubens Roxb., a remarkable plant native to the northeastern region of India, is notable for its wide array of medicinal properties, including antioxidant, antidiabetic, and immunomodulatory effects [6-8]. The essential oil extracted from the roots of Z. rubens is particularly rich, comprising 24 distinct compounds. Among these, monoterpenes dominate the profile, accounting for a substantial 75.3% of the oil, with (Z)-citral being the most prominent at 30.1%. Other significant monoterpenes present include camphene at 9.7%, β-phellandrene at 7.5%, and 1,8-cineole at 7.0%. Additionally, sesquiterpenoids are also identified, with zingiberene standing out as the most noteworthy at 5.3% [8].

The leaves of Z. rubens contribute to its therapeutic potential as well, containing a plethora of bioactive compounds such as reducing sugars, anthraquinones, terpenoids, flavonoids, saponins, tannins, alkaloids, and cardiac glycosides. Preliminary research suggests that the high concentrations of flavonoids and phenolic compounds in the plant may play a crucial role in its ability to scavenge free radicals, thus demonstrating significant antioxidant activity [8]. In our investigation, we performed an in vitro free radical scavenging assay, where we achieved noteworthy IC50 values, underscoring the plant's effectiveness in combating oxidative stress as detailed in the results.

In vitro studies do not always provide an accurate prediction of a substance's effectiveness in a living organism (in vivo) [19-21]. In our investigation, we noted that a specific plant extract exhibited moderate inhibitory activity against certain enzymes in laboratory settings. To further explore its potential as an anti-diabetic agent, we conducted experiments using animal models. The results revealed that this plant extract significantly decreased oxidative stress levels and offered protective benefits to the pancreatic beta cells in rats. While monitoring hemoglobin A1c (HbA1c) levels is crucial for assessing diabetes management, it is important to recognize that a reduction in these levels does not necessarily correlate with a meaningful decline in the microvascular complications commonly associated with diabetes [22-23].

Multiple signaling pathways have been identified as pivotal players in the onset and progression of diabetes and its associated complications. In particular, patients who receive a delayed diagnosis or those attempting to manage their blood glucose levels with oral hypoglycemic medications often exhibit elevated levels of Protein Kinase C (PKC). This increase in PKC activity activates Transforming Growth Factor Beta (TGF-β) along

with its related signaling pathways, setting off a cascade of events that leads to various diabetic complications.

Emerging evidence suggests a preliminary hypothesis in which the regulation of TGF-β by PKC may be implicated in the development of complications such as retinopathy, nephropathy, and neuropathy. To delve deeper into the molecular mechanisms underpinning these effects, we undertook a detailed investigation examining how HAZR affects the inhibition of PKC phosphorylation in mouse peritoneal macrophage cells [9-10].

Protein kinase C (PKC) plays a pivotal role in the phosphorylation process of AMP-activated protein kinase (AMPK), a key player in regulating endocrine functions within the body. Our research has demonstrated that HAZR, a compound of interest, significantly inhibits the phosphorylation of PKC when cells are challenged with Lipopolysaccharide (LPS), a known inflammatory agent. Notably, both LPS and Streptozotocin (STZ) equally promote inflammatory responses in the cellular environment, underscoring the complexity of these interactions [12-14].

The observed inhibition of PKC phosphorylation in our cultured cells suggests an important mechanism by which oxidative stress may influence PKC activity and the regulation of TGF-β, particularly in individuals suffering from diabetes. This insight is crucial for a deeper understanding of the multifaceted relationships that exist within diabetic pathology [15-16].

Additionally, the use of traditional medicine has stood the test of time as a global approach to managing various health conditions. These time-honored remedies often contain bioactive compounds that offer therapeutic benefits. However, the challenge lies in managing chronic diseases due to the potentially severe side effects associated with conventional treatments. This situation creates a pressing need for exploring alternative therapies that can effectively improve health outcomes and hinder disease progression [18-19].

In light of this, our current study has revealed that HAZR exhibits a notable anti-diabetic effect in diabetic animal models. This effect is achieved through mechanisms involving oxidative stress-mediated regulation of PKC and TGF-β, providing promising avenues for therapeutic intervention in diabetic conditions [21-32]. Here are the conclusions presented point by point:

1. Traditional medicine has been employed worldwide for centuries as a means to address various health issues, with many of these remedies containing potent bioactive compounds that offer therapeutic benefits. However, effectively managing chronic diseases can prove difficult due to the significant and often harsh side effects these treatments may cause.
2. Research findings revealed that HAZR has a substantial effect in inhibiting the phosphorylation of protein kinase C (PKC) when cells are stimulated by lipopolysaccharides (LPS), indicating its potential as a therapeutic agent.
3. Both PS (presumably a reference to a specific substance) and STZ (streptozotocin) exert similar inflammatory effects within the microenvironment of cells. Consequently, the observed inhibition of PKC phosphorylation in cell culture studies suggests a potential mechanism for the regulation of PKC and TGF-β associated with oxidative stress in individuals suffering from diabetes.
4. In diabetic animal models, HAZR exhibited notable antidiabetic properties, demonstrating its ability to regulate PKC and TGF-β through pathways influenced by oxidative stress. This highlights the potential of HAZR as a valuable therapeutic option for managing diabetes and its associated complications.

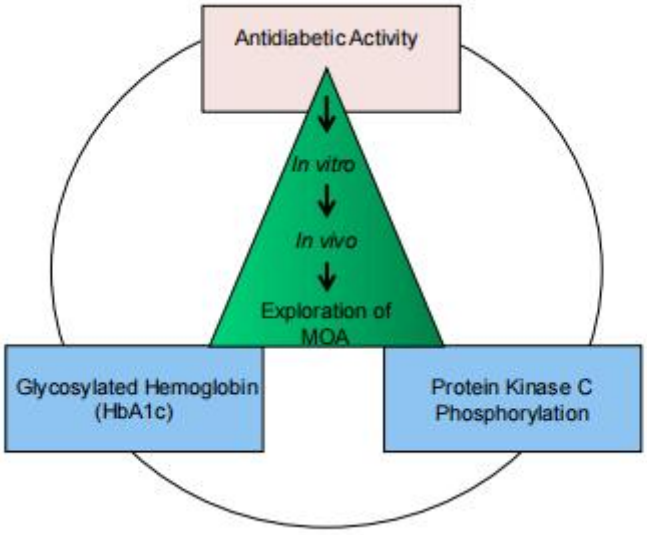

Figure 02: Schematic conclusion

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Sujit Das
25/11/24.

Plant Authentication Certificate

भारतसरकार
GOVT. OF INDIA
पर्यावरण, वनएवंजलवायुपरिवर्तनमंत्रालय
Ministry of Environment, Forest and Climate Change
भारतीयवनस्पतिसर्वेक्षण / Botanical Survey of India
सिक्किमहिमालयनक्षेत्रीयकेन्द्र/Sikkim Himalayan Regional Centre
गान्तोक - 737103 Gangtok -737103/ सिक्किम - SIKKIM

Phone: (O) 204717/2027898 203005
Fax.: 03592-204717
E-mail:gangtokbsishrc@gmail.com

संख्यासि.हि.क्षे.के./SHRC-5/02/2023-24/Tech./272 दिनांक/Dated: 27.09.2023

To,
Shri Sujit Das
Jadavpur University
Kolkata

Sub.: **Identification and Authentication of Plant Specimens** - reg.

Sir,

With reference to your letter no. NIL dated 29.08.2023, along with 1 no. of plant specimens collected from North East India have been identified and authenticated as follows:

Sl. No.	Collection No.	Scientific Name	Family
1.	JU-007	***Zingiber rubens*** Roxb..	ZINGIBERACEAE

Receipt of Rs. 500/- (Rupees five hundred only) with transaction reference No. 2609230005103 dated 26.09.2023 is enclosed herewith.

The above mentioned specimens are returned herewith.

Yours faithfully,

(जे. एच. फ्रेंक्लिन बेंजामिन/J.H. Franklin Benjamin)
वैज्ञानिक – घ एबं प्रभारी/Scientist- D & In-Charge

IAEC Approval Certificate

যাদবপুর বিশ্ববিদ্যালয়
কলকাতা-৭০০ ০৩২, ভারত

*JADAVPUR UNIVERSITY
KOLKATA-700 032,INDIA

PHARMACEUTICAL TECHNOLOGY
FACULTY OF ENGINEERING & TECHNOLOGY

Certificate

This is to certify that the project proposal no. **JU/IAEC-22/47** entitled **"Antidiabetic activity of plants available in north-east region"** submitted by **Prof. Pallab Kanti Haldar** has been approved/recommended by the IAEC of Department of Pharmaceutical Technology, Jadavpur University, in its meeting held on 15.06.2023 and 30 Wistar rats has been sanctioned under this proposal for a duration of next 12 months.

Authorized by	Name	Signature	Date
Chairman:	Prof. Sanmoy Karmakar	Sanmoy Karmakar	15/6/23
Member Secretary:	Prof. Pallab Kanti Haldar	Pallab Kanti Haldar	15/06/2023
Link Nominee of CPCSEA	Dr. Indranil Samanta	Indranil Samanta	15/06/2023

* Established on and from 24th December, 1955 vide Notification No.10986-Edn/IU-42/55 dated 6th December, 1955 under Jadavpur University Act, 1955 (West Bengal Act XXXIII of 1955) followed by Jadavpur University Act, 1981 (West Bengal Act XXIV of 1981)

দূরভাষঃ ২৪৫৭২৪৫৫ Website : www.jadavpur.edu Phone : 24572455

Poster Presentation

Among all the lifestyle disorders, most importantly overweight and obesity have become a global public health issue. To date, there are around 300 million obese worldwide and this increasing rate is alarming for us. Obesity is characterized by an increase in body weight that results in excessive fat accumulation, and forms a major underlying factor for the pathogenesis of several diseases like metabolic syndrome, Type 2 diabetes mellitus, cardiovascular and liver diseases and cancer. All of these pathological conditions are associated to oxidative stress (OS). Obesity is characterized by increase in adipose cell size, with aggravated local and systemic production of pro-inflammatory adipocytokines which induce increased OS. Ginger (Zingiber officinalis) is known to have important properties like antioxidant, anti-inflammatory, and anti-obesity effects. The ginger is modulating adipocyte metabolism but the mechanism remained unexplored. Majorly the active polyphenolic compounds play various roles to control the parameters like body weight, BMI, kidney, liver, and atherogenic indices. The present study is dedicated on the detailed mechanism underlying the metabolic impact of Zingiber officinalis in obesity and obesity-related diseases. The antioxidant effects of ginger with regards to the role on oxidative stress induced obesity and conditions are found to be very promising. Attention to these natural compounds would open a new approach for novel therapeutic and more effective agents.

SNPS-JU-SEM-23/113

Anti-diabetic Activity of a medicinal Plant Available in North-East Region of India

Sujit Das

Jadavpur University, Kolkata.

Traditionally used medicinal plants were the source of abundant indigenous active substances, which are being used by the people of different communities around the globe for different ailments. The north-eastern region of India is highly resourceful in terms of traditionally used medicinal plants for different diseases and disorders. Among these, diabetes is one of the most common disorders; prolonged use of its medication leads to various adverse effects. In respect to this situation, it will urge more research on effective antidiabetic drugs from medicinal plants. Zingiber rubens Roxb. (Family: Zingiberaceae) is such a miraculous plant, traditionally used in different ailments by the locals of Meghalaya, in the north-east region of India. After performing antioxidant activity (DPPH scavenging assay, NO scavenging assay, superoxide scavenging assay, and hydroxyl radical scavenging assay) and in-vitro antidiabetic activity (% inhibition of -amylase, % inhibition of -glucosidase) of the root extract of Zingiber rubens Roxb. (Family: Zingiberaceae), it was found that they have these mentioned properties and also may have other medicinal properties.

Poster Presentation

National Seminar on

"Role of Medicinal plants to combat metabolic disorders and associated pathological complications"

School of Natural Product Studies

Jadavpur University, Kolkata, India

June 30 – July 01, 2023

Certificate

This to certify that Sujit Das **participated & presented a paper in the Poster session in the National Seminar held at Jadavpur University, Kolkata 700 032, India on June 30 – July 01, 2023.**

Prof. Chiranjib Bhattacharjee
Pro Vice Chancellor
Jadavpur University

Prof. Snehamanju Basu
Registrar
Jadavpur University

Prof. Pallab Kanti Haldar
Organizing Secretary
National Seminar - 2023

Poster Presentation

Poster Presentation

POSTER NO: GCP-P002

Exploration of the Molecular Mechanism Underlying the Antidiabetic Activity of a North East Indian Plant *Zingiber Rubens* Roxb. through Modulation of Pkc Phosphorylation

Sujit Das[1,2], Piyali Devroy[3], Suraj Kumar Chatterjee[3], Sandipan Jana[1,2], Srijon Gayen[1,2], Suparna Ghosh[1,2], Manas Bhowmik[1], Asis Bala[3], Pallab Kanti Haldar[1,2,*]

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ABSTRACT:

Background: *Zingiber rubens* Roxb., a new species of the Zingiberaceae family, is found profoundly in the Northeastern region of India. It is a rhizomatous geophyte and grows primarily in the seasonally dry tropical biome native to Indo-China regimens.

Objective: The aim is to research on the root part of this plant to discover active constituents and evaluate the molecular mechanism of anti diabetic activity.

Methods: The hydroalcoholic root extract of this plant (HAZR) was evaluated for *in-vitro* antioxidant assays α-amylase, and, α-glucosidase inhibition assay. Further, the anti diabetic activity assay was evaluated exploring the molecular mechanism for modulation of oxidative stress in tissue biochemical parameters and HbA1c. To explore the molecular mechanism we also investigated the inhibition assay of protein kinase C (PKC) phosphorylation in mouse peritoneal macrophages.

Results: The results of the experiments helped to conclude that HAZR has potent antioxidant and anti-diabetic activity; also, could have been embedded with potent active molecules.It also reveled the mechanism of action as inhibition of PKC phosphorylation meriting further studies.

Conclusion: The present study revealed that HAZR demonstrated its anti diabetic activity against diabetic animal model through oxidative stress-mediated PKC and TGF-β regulation in diabetic individuals.

Keywords: *Zingiber rubens* Roxb.; antidiabetic activity; Modulation of oxidative Stress; Inhibition of Protein Kinase C, HbA1c.

2

Publications

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Authors:
Sujit Das, Piyali Devroy, Suraj Kumar Chatterjee, Sandipan Jana, Srijon Gayen, Suparna Ghosh, Manas Bhowmik, Asis Bala and Pallab Kanti Haldar

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RESEARCH ARTICLE

Exploration of the Molecular Mechanism Underlying the Antidiabetic Activity of a Northeast Indian Plant *Zingiber rubens* Roxb. through Modulation of PKC Phosphorylation

Sujit Das[1,2], Piyali Devroy[3,4], Suraj Kumar Chatterjee[3], Sandipan Jana[1,2], Srijon Gayen[1,2], Suparna Ghosh[1,2], Manas Bhowmik[1], Asis Bala[3,4,*] and Pallab Kanti Haldar[1,2,*]

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***Objective*:** The aim is to research the root part of this plant to discover active constituents and evaluate the molecular mechanism of antidiabetic activity.

***Methods*:** The hydroalcoholic root extract of this plant (HAZR) was evaluated for *in-vitro* antioxidant assays α-amylase and α-glucosidase inhibition assay. Further, the antidiabetic activity assay was evaluated, exploring the molecular mechanism for modulating oxidative stress in tissue biochemical parameters and HbA1c. To explore the molecular mechanism, we also investigated the inhibition assay of protein kinase C (PKC) phosphorylation in mouse peritoneal macrophages.

***Results*:** The experiments' results helped to conclude that HAZR has potent antioxidant and antidiabetic activity and could have been embedded with potent active molecules. They also revealed the mechanism of action as inhibition of PKC phosphorylation, meriting further studies.

***Conclusion*:** The present study revealed that HAZR demonstrated its antidiabetic activity against diabetic animal models through oxidative stress-mediated PKC and TGF-β regulation in diabetic individuals.

Keywords: *Zingiber rubens* roxb., antidiabetic activity, modulation of oxidative stress, inhibition of protein kinase C, HbA1c, PKC.

1. INTRODUCTION

Traditional medicinal plants have been gaining interest in recent times due to their potential to prevent and cure various ailments [1, 2]. These plants contain indigenous active substances that have been used by people from different communities around the world for different diseases [3, 4]. The northeastern region of India is rich in traditional medicinal plants used for different diseases and disorders [2]. Diabetes is one of the most common disorders, and the prolonged use of medication leads to various adverse effects [5]. Therefore, more research is needed to find effective antidiabetic drugs from medicinal plants.

Zingiber rubens Roxb. is a plant commonly found in Meghalaya, a state in the northeastern region of India [6]. It is traditionally used for different ailments by the locals. The plant consists of a leafy stem 4-6 feet tall with rhizomes [6, 7]. The rhizomes of the plant can propagate 6-8 plants in a slow process. This plant was collected from the Meghalaya province and is also being grown in our institution's medicinal garden [7, 8]. The most significant components of *Z. rubens* are camphene (9.7%), Z-citral (30.1%), 1,8-cineole (7.0%), and β -phellandrene (7.5%). This plant has anti-inflammatory and antioxidant activities because of the presence of 1,8-cineole in its active constituents. It could have other activities and active molecules as well [8].

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Zingiber rubens is a sub-Himalayan species of plant belonging to the family Zingiberaceae [6]. It has distinct red-colored flowers and a tuberous root [7]. It is commonly known as "Bengal Ginger" and is distributed throughout Southeast Asia, including Northeast India, Bangladesh, Myanmar, Thailand, and Vietnam. Although little research has been done on this plant, it has been used in folk medicine to treat cough, cold, infection, fever, diarrhea, and more [6-8]. A total of at least 24 compounds have been identified from the root oil of *Z. rubens,* comprising largely of monoterpenes (75.3%) dominated by (Z)-citral (30.1%), camphene (9.7%), β-phellandrene (7.5%), and 1,8-cineole (7.0%). Zingiberene (5.3%) was significant among the sesquiterpenoids, while the leaves showed phytochemical constituents such as reducing sugar, anthraquinone, terpenoids, flavonoids, saponins, tannins, alkaloids, cardiac glycosides [9, 10]. One study has shown that Methanolic extract of the leaves of *Z. rubens* has effective antidiarrheal activity *in vivo* and thrombolytic effect *in vitro* [11, 12]. However, the plant phytochemicals have not shown any antibacterial activity as claimed by the folk use of this plant.

This study aims to assess the traditional claim that this plant has antidiabetic properties. Additionally, we investigated the molecular mechanism of this plant's antidiabetic activity by examining its effects on redox oxidative stress pathways and a protein kinase C (PKC) inhibition assay.

2. MATERIALS AND METHODS

2.1. Plant Materials

An authenticated dealer collected the mature rhizomes/ roots of the plant from the local market. The Botanical Survey of India, Gangtok, Sikkim, Gangtok 737103, India, further authenticated the plant material. The certificate of authentication (SHRC-5/02/2023-24/Tech/272) was securely stored at the School of Natural Product Studies, specifically at the Department of Pharmaceutical Technology, which is located at Jadavpur University in Kolkata, West Bengal, India [1, 4].

2.2. Extraction of the Plant Materials

The fresh rhizomes and roots were collected, dried, ground to a smaller size, and sifted through a mesh with a size of 100 [12, 13]. The resulting crudes were packed into a thimble and placed in a Soxhlet apparatus with a mixture of hydroalcoholic solvents (70% methanol and 30% water). The hydroalcoholic extract of *Zingiber rubens* (HAZR) was collected, dried, and stored in an airtight container at 4°C for further use in the experiment [1, 4, 14].

2.3. *In vitro* Free Radical Scavenging Assay

2.3.1. DPPH Scavenging Assay

To estimate the DPPH radical scavenging activities of different extracts, the 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma Cat. NO. D9132; ≤ 100 % pure) assay method was used [15]. A methanolic solution of 0.1 mM DPPH was prepared along with samples of varying concentrations (25, 50, 100, 200, 400, and 600 µg/ml). The agitation speed of 100 SPM (strokes per minute) and λ_{max} =517 nm were considered during the assay. Standard solutions of varying concentrations (4, 8, 12, 16, and 20 µg/ml) of ascorbic acid were also prepared. The reaction was initiated by adding 1 ml of the DPPH solution to 3 ml of extract in methanol. The mixture was agitated vigorously and kept in a dark area at room temperature for 30 minutes until the reaction was completed. The absorbances were measured at a wavelength of 517 nm using an ultraviolet spectrophotometer. The % inhibition was calculated using the following formula.

% inhibition of DPPH= ([A - C/ C] × 100), whereas, A=absorbance of control and C= absorbance of sample/extract reaction.

2.3.2. Hydroxyl Radical Scavenging Assay

Hydroxyl radical is a highly reactive oxygen species that can cause damage to cell membrane phospholipids by reacting with polyunsaturated fatty acids. The reaction mixture (1.0 mL) includes 2-deoxy-D-ribose (28 mM in 20 mM KH_2PO_4-KOH buffer, pH 7.4) 100 µL, extract 500 µL, EDTA (1.04 mM) 200 µL, $FeCl_3$ (1:1 v/v) 200 µM, H_2O_2 (1.0 mM) 100 µL, and ascorbic acid (1.0 mM) 100 µL. This mixture is then incubated at 37°C for 60 minutes. Thiobarbituric acid (1%) 1.0 mL and trichloroacetic acid (2.8%) 1.0 mL are added to the mixture, which is then incubated at 100°C for 20 minutes. After cooling, the absorbance is measured at 532 nm against a blank sample. The % inhibition is calculated using the following formula [15, 16].

% inhibition of DPPH= ([A - C/ C] × 100), whereas, A=absorbance of control and C= absorbance of sample/ extract reaction.

2.3.3. Nitric Oxide/ NO Scavenging Assay

Nitric oxide synthases found in biological tissues are responsible for the conversion of arginine to citrulline, leading to the formation of NO *via* a five-electrode oxidative reaction. When sodium nitroprusside decomposes in aqueous solutions at pH 7.2, it produces NO. When reacting with oxygen under aerobic conditions, it forms stable products like nitrate and nitrite. To determine their quantities, Griess reagents can be used. The procedure involves mixing 2 mL of sodium nitroprusside (10 mM) dissolved in 0.5 mL phosphate buffer (pH 7.4) with 0.5 mL of extract at different concentrations (0.2–0.8 mg/mL). The mixture is then incubated at 25°C for 150 min, after which 0.5 mL of the incubated solution is mixed with 0.5 mL of Griess reagent (1.0 mL sulfanilic acid reagent). The mixture is then incubated again for 30 min at room temperature, and absorbances are measured at 546 nm. Finally, the amount of nitric oxide radical inhibition can be calculated using the provided equation [15, 16].

$$\% \text{ inhibition of NO radical} = [A_0 - A_1]/A_0 \times 100$$

Whereas A_0: Absorbance before reaction and A_1: absorbance after reaction occurred with Griess reagent.

2.3.4. Superoxide Scavenging Assay

Superoxide anion is a weak oxidant that produces harmful hydroxyl radicals and singlet oxygen, leading to oxidative stress. In this method, superoxide radicals were generat-

ed in a solution of 3.0 mL Tris-HCl buffer (pH 8.0) containing 0.5 mL nitro-blue tetrazolium (NBT), 0.5 mL NADH solution, 1.0 mL extract and 0.5 mL Tris-HCl buffer (pH 8.0). The reaction was initiated by adding 0.5 mL phenazine methosulfate (PMS) solution (0.12 mM) to the mixture, which was then incubated at 25°C for 5 min. The absorbance was measured at 560 nm against a blank sample. The % inhibition is calculated using the following formula [15, 16].

% inhibition of DPPH= ([A - C/ C] × 100), whereas, A=absorbance of control and C= absorbance of sample/ extract reaction.

2.4. α-amylase Inhibitory Activity

This activity was measured according to the method described by Jana *et al*., 2023 [1], with some small changes. Acarbose was used as the standard control. To prepare the starch solution, 1 g of starch was dissolved in 50 mL of NaOH and heated at 100°C for 5 minutes. The solution was then cooled using iced water, and the pH was adjusted to 7 by adding 2 M HCl. Finally, the solution was made up to 100 mL by adding H_2O.

To prepare the sample, 10 mg of extract was added to 1 mL of MeOH: H_2O (1:1). In order to achieve complete enzyme inhibition, dilutions of PBS (Phosphate Buffered Saline) were prepared by mixing 35 µL of PBS, 35 µL of substrate, and 5 µL of sample in a 96-well microtiter plate. The plate was then incubated at 37°C for 1 minute. After that, 20 µL of 50 mg/mL α-Amylase solution was added to each well and incubated for 15 minutes. The reaction was stopped with 50 µL of 0.1 M HCl and 150 µL of 0.5 mM iodine solution. The absorbance was measured at 580 nm. % inhibition of α-Amylase = [1 − (ABS2 − ABS1) / (ABS4 − ABS3) × 100]

Here, ABS_1: Absorbance of an incubated solution containing sample, starch and amylase

ABS_2: Absorbance of an incubated solution containing sample and starch

ABS_3: Absorbance of an incubated solution containing starch and amylase, and

ABS_4: Absorbance of an incubated solution containing starch

IC_{50} value was calculated by interpolating using Graph Pad Prism software version 5 considering force zero (X=0 and Y=0)

2.5. α-glucosidase Inhibition Assay

To measure the activity, a 96-well microtiter plate was used. The substrate used was p-nitrophenyl-α-D-glucopyranoside. The method used was by Jana *et al*., 2023 [1] with slight modifications. A sample was prepared by adding 10 mg extract to 1 mL of MeOH: H_2O (1:1). Dilutions of PBS (Phosphate Buffered Saline) were then made. To carry out the reaction, 75 µL of PBS was mixed with 5 µL of the sample and 20 µL of enzyme solution. The mixture was pre-incubated at 37°C for 5 minutes before the reaction was initiated by adding the substrate. The amount of p-nitrophenol (p-NP) released was measured in a microplate reader at 405 nm for 1 hour, with absorbance readings taken every 5 minutes. Acarbose was used as the standard control. % inhibition of α-Glucosidase= $[(A_0 - A_S) /A_0] \times 100$.

Here, A_0: Absorbance recorded for the control (enzymatic activity without inhibitor); A_S: Absorbance recorded for the sample (enzymatic activity in the presence of the inhibitor). IC_{50} value was calculated by interpolating using Graph Pad Prism software version 5 considering force zero (X=0 and Y=0).

2.6. Experimental Animal Model

For this study, we used adult male Wistar albino rats (weighing 160-180 g) provided by M/S Chakraborty Enterprise, Kolkata (Reg. no. 1443/PO/Bt/s/11/CPCSEA). The rats had fasting blood glucose (FBG) levels between 85-90 mg/dL and were given an acclimatization period of 7 days with unlimited access to food and water. The rats were housed in a room kept at 25°C with a 12-hour light/ dark cycle and were given unrestricted access to water and a standard high-fat diet. Animal experimentation protocols were thoroughly examined and approved by the Animal Ethical Committee at Jadavpur University in Kolkata, West Bengal, India. The University Animal Ethics Committee approved the protocol with the approval number JU/IAEC-22/47 on June 15th, 2023.

M/s produced the Animals. Chakraborty Enterprise, 3/1D, Girish Vidyaratna Lane, Narikeldanga, Kolkata-700011, West Bengal, India, with Govt. of India (CCSEA) Approval Number 1443/PO/Bt/S/11/CPCSEA.

The experiment was conducted on 30 Rats, whereas n=6 rats were considered for each experiment group.

- Sample Size=30 (n=6; five groups)- grouping was shown in the accepted manuscript section- 2.6.2. Animal model.

2.6.1. Oral Glucose Tolerance Test (OGTT)

The study involved rats that had not eaten overnight and had normal blood sugar levels (between 85-90 mg/dl) [1, 4]. Six rats were placed in each of the three groups. The rats in groups II and III were given HAZR (at doses of 200 and 400 mg/kg b.w., respectively), while the rats in group I were given only distilled water (5 mL/kg b.w. p.o.) and acted as the control group. After this, all groups were given oral glucose (2 g/kg b.w.). Blood samples were taken from the tail vein just before and 30, 60, 120, and 240 minutes after oral glucose delivery. Blood glucose levels were measured using an Accu-Chek glucometer. The study was conducted followed by the methods of Jana *et al*., 2023 [1].

2.6.2. Animal Model

The toxicity of HAZR was examined through an acute oral toxicity study that followed the recommendations of the Organisation for Economic Co-operation and Development (OECD) 425 guidelines from 2008. The experiment involved a 28-day high-fat diet treatment followed by a 14-day duration study. Six rats were assigned to each of the five groups, making a total of 30 rats [1, 4].

- ✓ **Group –I**: Normal control group: The rats received normal saline (0.5 ml/kg, p.o.) for 14 days.
- ✓ **Group-II**: Diabetic (STZ+HFD) control group: The diabetic group consisted of rats treated with STZ (35 mg/kg, b.w., *i.p*.) and a high-fat diet.
- ✓ **Group-III:** HAZR Extract (HAZR 200 mg/kg) treated group: The diabetic rats were treated orally with HAZR (200 mg/kg b.w.) for 14 days.
- ✓ **Group-IV:** HAZR Extract (HAZR 400 mg/kg) treated group: The diabetic rats were orally treated with HAZR (400 mg/kg b.w.) for 14 days.
- ✓ **Group-V:** Metformin-treated group: The diabetic rats were treated with metformin (150 mg/kg, p.o.) for 14 days.

The next day after the last dose of HAZR, all animals were sacrificed, and the required blood and tissue samples were collected for further analysis [1-4].

2.6.3. Estimation of Glycosylated Hemoglobin Level (HbA1c)

Using a commercially available test kit (Coral Clinical System, Tulip Diagnostics Pvt. Ltd. India), the amount of glycosylated hemoglobin was measured by analyzing a complete blood sample. This assay methodology involves the use of an ion exchange resin.

2.6.4. Estimation of Serum Biochemical Parameters

Collected serum was examined using a commercially available assay kit for various biochemical factors, including total protein, serum alkaline phosphatase (SALP), serum glutamic pyruvic transaminase (SGPT), and serum glutamic oxaloacetic transaminase (SGOT), provided by Arkray Healthcare Pvt. Ltd. in India [1, 4].

2.6.5. Serum Lipid Profile

Using commercially available assay kits from Arkray Healthcare Pvt. Ltd. in India, we measured the serum lipid profiles of HFD-STZ-induced diabetic rats, including levels of total cholesterol, HDL cholesterol, and triglycerides [1, 4].

2.6.6. Histopathological Studies

For histopathological investigations, researchers collected pancreatic tissue from sacrificed rats. The tissue was washed in normal saline and then fixed in 10% formalin for at least 24 hours. After that, it was dehydrated with alcohol, embedded in paraffin, cut into 4 to 5-micrometer-thick sections, and stained with hematoxylin and eosin dye [1].

2.7. Protein Kinase C and Phospho-Protein Kinase C Estimation Assay

Mouse Peritoneal Macrophages (Cellbiologics; Catalog No. 5032TF) were grown in RPMI medium supplemented with 10 % FBS in 5% CO_2 and controlled conditions. More than 95% of viable cells were treated with or without LPS (25 µg/ml) and different concentrations of HAZR (0-100 µg/ml) in the same controlled condition in 96 well plates. The PKC and Phospho-PKC levels were estimated using a commercially available Sandwich ELISA kit (KNH11806 and KLM0591, respectively).

2.8. Statistical Analysis

The mean and SEM are used to express all results. Graph Pad Prism 8.02 software was used to perform a one-way analysis of variance (ANOVA) and post hoc Dunnett test to evaluate whether the results were statistically significant (Graph Pad Software, USA). Statistics were considered significant at $p < 0.05$ [1, 4].

3. RESULT

3.1. Identification and Authentication of the Plant Materials

The plant material was authenticated by the Botanical Survey of India, Gangtok, Sikkim, Gangtok 737103, India. The certificate of authentication (SHRC-5/02/2023-24/Tech/272) was securely stored at the School of Natural Product Studies, specifically at the Department of Pharmaceutical Technology, which is located at Jadavpur University in Kolkata, West Bengal, India.

3.2. Extraction of the Plant Materials

Hydroalcoholic extract of *Zingiber rubens* (HAZR) was prepared using a Soxhlet apparatus with a mixture of hydroalcoholic solvents (70% methanol and 30% water). The plant parts used were rhizomes and roots. The hydroalcoholic extract of *Zingiber rubens* (HAZR) was collected, dried, and stored in an airtight container at 4°C and further used for the experiment.

3.3. *In vitro* Free Radical Scavenging Assay

3.3.1. DPPH Scavenging Activity

The DPPH radical scavenging activity of HAZR was estimated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay method using ascorbic acid as a standard. The findings are expressed as % inhibition of DPPH against concentration in Fig. (**S1**). We found HAZR to be effective in scavenging free radicals, with an IC_{50} of 25 µg/ml, whereas the standard shows an IC_{50} value of about 42 µg/ml (Fig. **1**).

3.3.2. Nitric Oxide/NO Scavenging Activity

The Griess reagent estimated the nitric oxide radical inhibition of the extract at different concentrations (0.2–0.8 mg/mL), and the percentage inhibition of the NO radical was calculated and plotted against concentration (Figs. **S2** and **S3**). Ascorbic acid was used as a standard. We found HAZR to be effective, with an IC_{50} value of 38 µg/ml (Fig. **1**).

3.3.3. Hydroxyl Radical Scavenging Activity

The hydroxyl radical activity of HAZR was analyzed using the reaction mixture of 2-deoxy-D-ribose, extract, EDTA, Thio-barbituric acid, and trichloroacetic acid. Ascorbic acid was used as a standard. HAZR showed an inhibitory

half-maximal concentration (IC_{50}) at 23 µg/ml, which is compared with the standard IC_{50} value of almost 39 µg/ml for Ascorbic acid (Figs. **1** and **S4**).

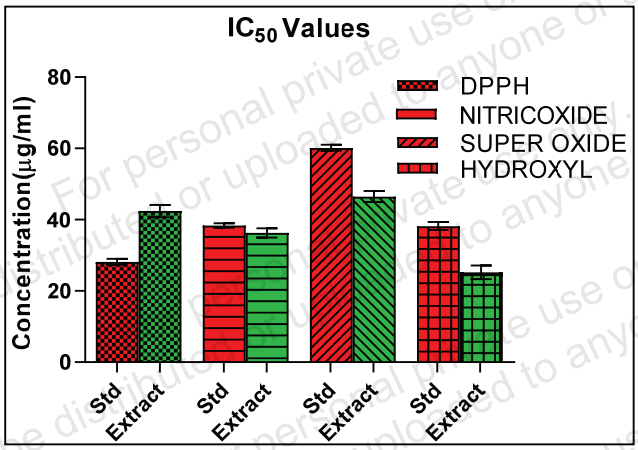


Fig. (1). Comparative IC_{50} values of *in vitro* free radical scavenging assay. Values are expressed as mean ±SEM (n=3), and Vitamin C was used as standard (Std). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.3.4. Superoxide Scavenging Activity

Superoxide anion assay was conducted using nitro-blue tetrazolium (NBT) in Tris-HCl buffer, and phenazine methosulfate (PMS) solution was used to initiate the reaction. This was compared with a standard of Ascorbic acid. The finding showed the effectiveness of HAZR as an antioxidant at the IC_{50} value of 45 µg/ml, whereas the commonly used standard showed a value of 60 µg/ml (Fig. **1**).

3.4. α-amylase Inhibitory Activity

The *in-vitro* inhibitory activity of HAZR against α-amylase was measured as an inhibitory half-maximal concentration (IC_{50}) at different concentrations of the plant extract against the standard marker Acarbose. The inhibitory activity (IC_{50} value) of HAZR was found to be 373.6 ± 2.27 µg/ml and 317.1 ± 3.32 µg/ml for the standard drug Acarbose. This finding was almost equivalent to standard acarbose inhibition (Fig. **2A**).

3.5. α-glucosidase Inhibitory Activity

In-vitro inhibitory activity of HAZR against α-glucosidase was measured at different concentrations of the plant extract by calculating the IC_{50} value with the common comparable inhibitor drug Acarbose as standard. The IC_{50} value for α-glucosidase was 345.6 ± 5.63 µg/ml and 51.2 ± 1.63 µg/ml for the HAZR and the standard drug Acarbose, respectively (Fig. **2B**).

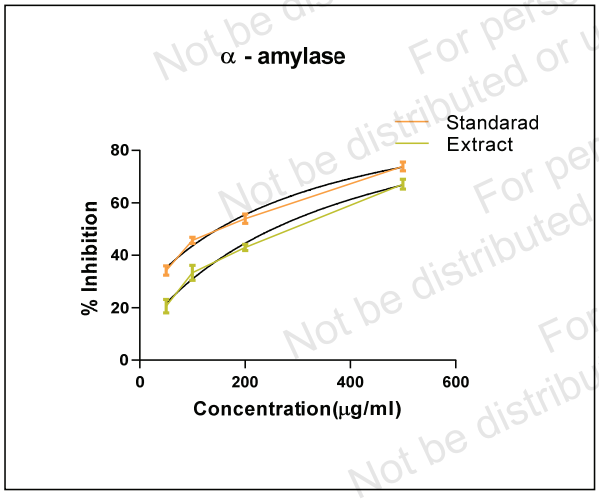
3.6. Experimental Animal Model

3.6.1. Oral Glucose Tolerance Test (OGTT)

Rats with normal blood sugar levels (85-90 mg/dl) underwent an oral blood glucose tolerance test. Following glucose injection, blood glucose levels increased during the first 30 minutes before steadily declining over the following 60, 90, and 120 minutes, as shown in Table **1**. HAZR at a dose of 400 mg/kg b.w. significantly reduced the glucose level in tested animals in OGTT.

3.6.2. Animal Model

Acute oral toxicity of HAZR was studied following the guidelines of OECD 425 from 2008. No toxicity was observed after the administration of HAZR up to 2000 mg/kg



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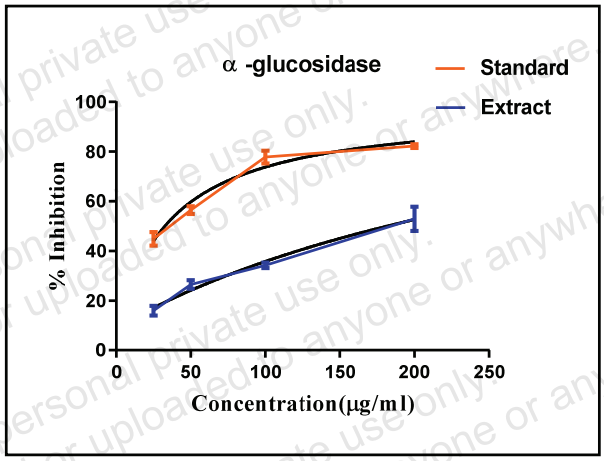


B

Fig. (2). % inhibition of α-amylase shown by different concentrations of Extract and Acarbose. (**A**) The IC_{50} value for α-amylase was 373.6 ± 2.27 µg/ml and 317.1 ± 3.32 µg/ml for the HAZR and the standard drug Acarbose, respectively. (**B**) The IC_{50} value for α-glucosidase was 345.6 ± 5.63 µg/ml, and 51.2 ± 1.63 µg/ml for the HAZR and the standard drug Acarbose, respectively. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Effect of HAZR on oral glucose tolerance test.

Groups	0 min	30 min	60 min	120 min
Normal control	89.83 ± 3.9	125.7 ± 2.9	115.7 ± 1.5	110.0 ± 2.08
LD- HAZR (200)	90.33 ± 1.2	126.0 ± 1.5	120.3 ± 1.8	105.7 ± 1.4
HD- HAZR (400)	85 ± 1.5	120.3 ± 1.2*	111.3 ± 2.4*	96.6 ± 2.02*
Metformin 150 mg/kg	81.5 ± 2.5	108 ± 2.30*	98.2 ± 3.20*	87 ± 1.80*

Note: Values are expressed as mean ± SEM (n=6). *Values significantly differ from normal control where $p < 0.05$.

Table 2. Effect of HAZR on fasting blood glucose (mg/dl).

Groups	Diabetic Animal (After 28th Days of HFD and Single dose STZ treatment)		
	Day 0	Day 7	Day 14
Normal control	76 ± 12.23	81.33 ± 11.80	88.66 ± 13.41
STZ (45 mg/kg)	410.33 ± 13.52a*	418.66 ± 12.19a*	515 ± 11.15a*
STZ + LD	422.63 ± 12.13	397.33 ± 11.75b*	338.41 ± 12.04b*
STZ + HD	409.33 ± 12.10	306.34 ± 11.45b*	216.33 ± 11.46b*
STZ + metformin (150 mg/kg)	405.31 ± 13.33	259.22 ± 12.10b*	108.66 ± 11.26b*

Note: Values are expressed as mean ±SEM (n=6). *Each values are expressed as mean±SEM (n=6), a*Diabetic control group versus normal control group ($p < 0.05$), b*All treated group versus diabetic control group on corresponding day ($p < 0.05$), *Values significantly differ from each other where $p < 0.05$. SEM: Standard error of mean, STZ: Streptozotocin.

b.w. For this experimental investigation, two doses of HAZR were selected: 200 mg/kg b.w. and 400 mg/kg b.w. as low dose and high dose, respectively.

3.6.3. Effect on Glycosylated Hemoglobin Level (HbA1c)

Low-dose STZ high-fat-induced diabetic rats had significantly ($p < 0.05$) higher FBG levels than the normal control group. When administered to diabetic rats for 14 days at 200 and 400 mg/kg doses, HAZR led to a substantial ($p < 0.05$) decline in FBG level towards normal compared to the diabetic control group. The effect of HAZR on Fasting Blood Glucose (mg/dl) is shown in Table **2**. Furthermore, HAZR-treated rats had significantly reduced levels of glycosylated hemoglobin ($p < 0.05$) compared to diabetic rats (Fig. **3**).

3.6.4. Estimation of Serum Biochemical Parameters

Compared to the low-dose STZ high-fat diet control group, HAZR-treated groups demonstrated a substantial ($p < 0.05$) decrease in blood biochemical markers such as SALP, SGOT, and SGPT. The total protein level increased in the treated group compared to the low-dose STZ high-fat diet control group (Figs. **4A**-**D**). Serum biochemical parameters indicated the hepatoprotective nature of HAZR in both low and high doses of the plant extract evidently.

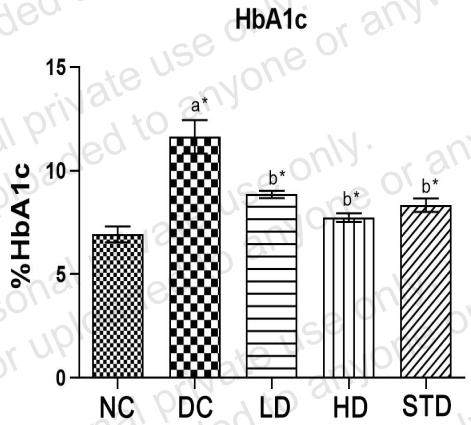


Fig. (3). Estimation of Glycosylated Haemoglobin (Hba1c). a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to diabetic control". (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

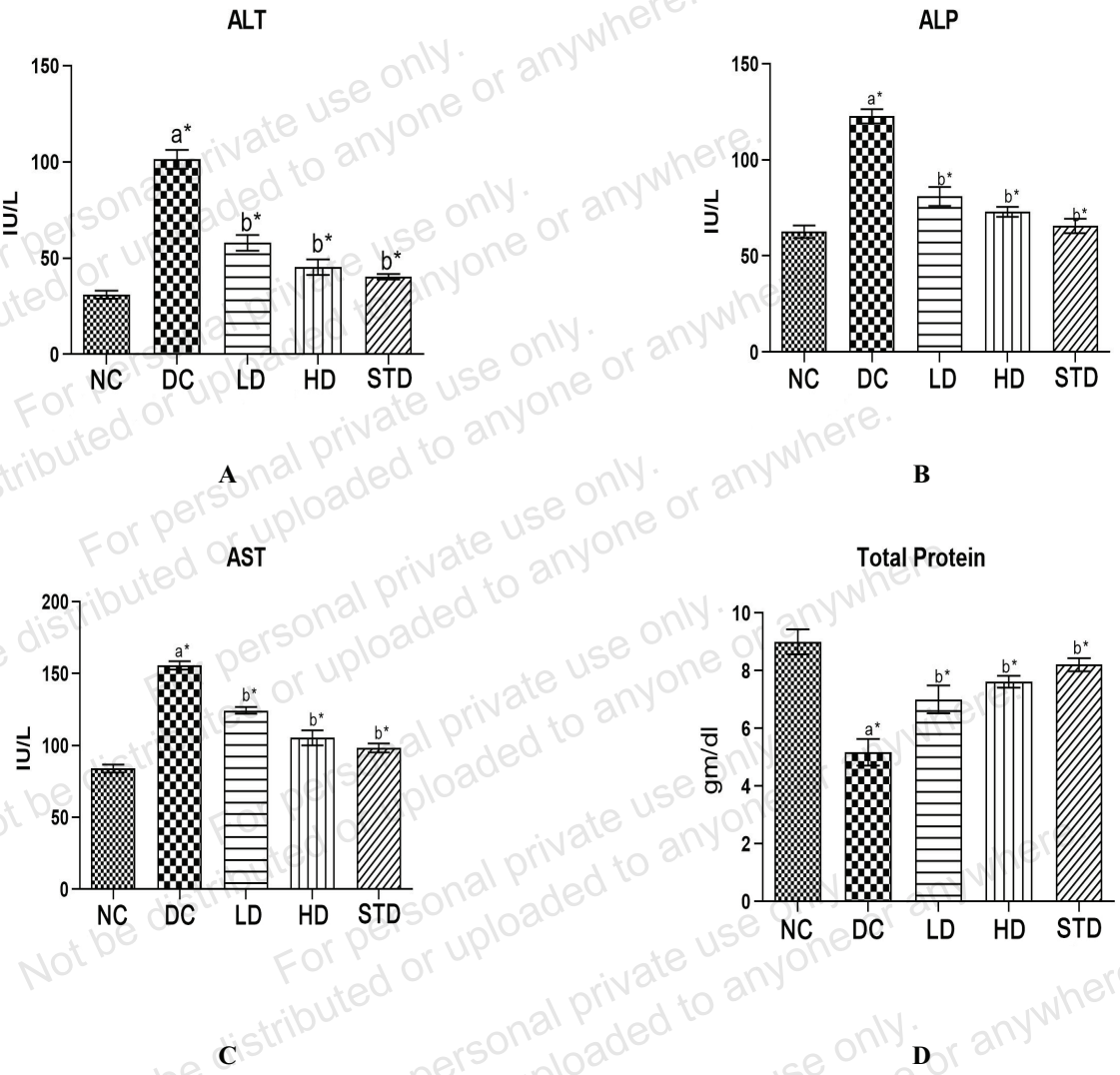


Fig. (4). Estimation of serum liver function test parameters. "Each value is expressed as Mean ± SEM where n=6. a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to diabetic control". (**A**) ALT Activity (IU/L). (**B**) ALP Activity (IU/L). (**C**) AST Activity (IU/L). (**D**) Total protein (gm/dl) (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.6.5. Serum Lipid Profile

The serum lipid profiles of diabetic rats had considerably ($p < 0.05$) higher triglycerides and total cholesterol and significantly ($p < 0.05$) lower HDL when compared to normal rats. In comparison to diabetic groups, triglyceride and total cholesterol levels were significantly ($p < 0.05$) decreased after HAZR extract administration at doses of 200 mg/kg and 400 mg/kg. In contrast, HDL levels were significantly ($p < 0.05$) elevated (Figs. **5A**-**C**).

3.6.6. Histopathological Studies

In HAZR-treated rats compared to diabetic rats, the MDA level was significantly ($p < 0.05$) decreased, SOD and GSH levels were significantly ($p < 0.05$) enhanced, and the malonaldehyde level was significantly ($p < 0.05$) decreased (Figs. **6A**-**C**). Studies on pancreatic histopathology revealed that groups treated with HAZR had pancreatic beta cell protective action. In comparison to diabetic rats, there was a dose-dependent gradual improvement in pancreatic beta cell density in groups treated with HAZR (Figs. **7A**-**E**).

3.7. Protein Kinase C and Phospho-protein Kinase C Estimation

To explore the molecular mechanism of action, we investigated the effect of HAZR on the inhibition of phosphorylation of PKC in mouse peritoneal macrophage cells. Protein kinase C (PKC) is important in phosphorylating AMP-activated protein kinase (AMPK) in endocrine control. HAZR significantly inhibited the phosphorylation of PKC when LPS stimulated the cell, this is evident by the gradual increase in PKC level at the different increasing concentrations of the plant extract. In contrast, the level of p-PKC is decreased depicting the inhibitory effect of HAZR on PKC phosphorylation (Figs. **8A**-**D**).

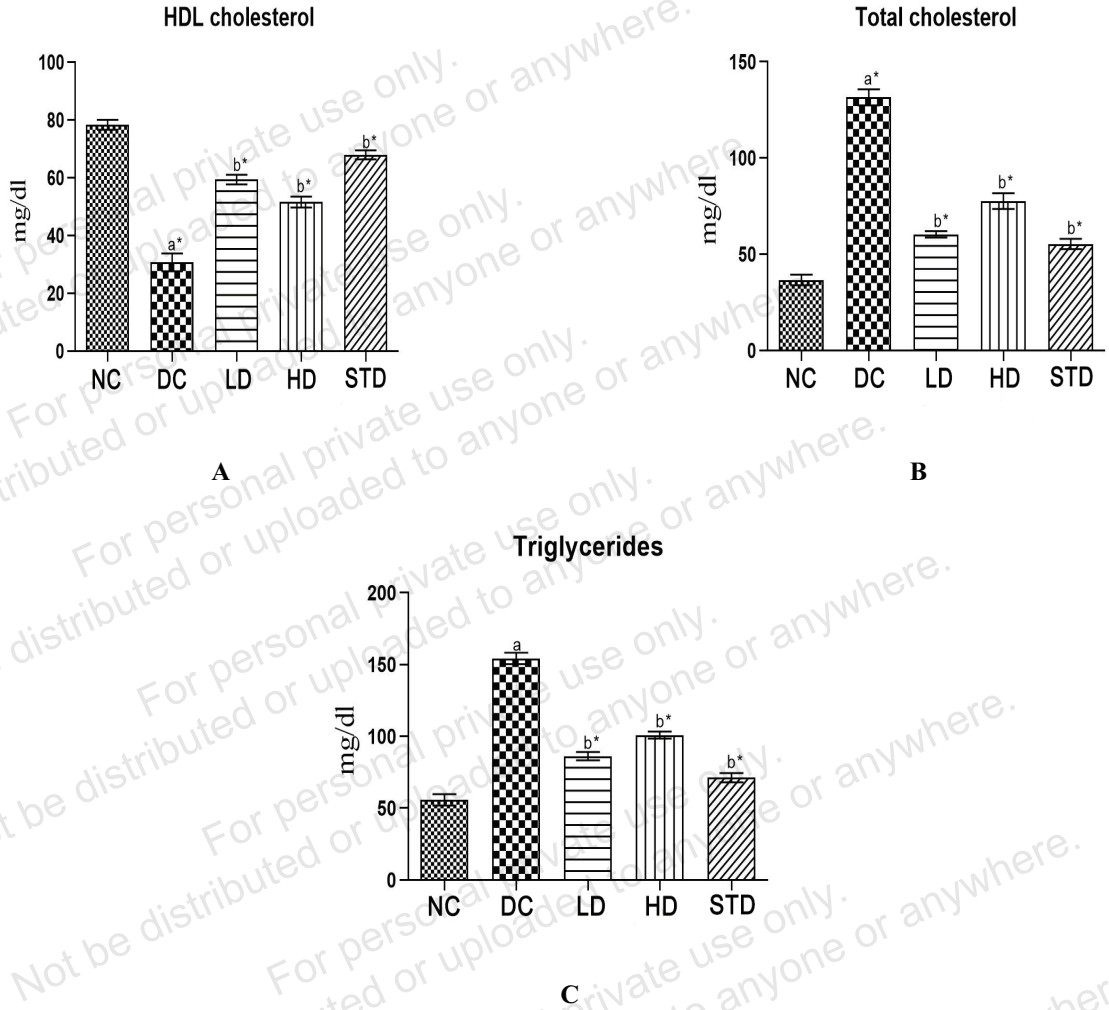


Fig. (5). Estimation of serum lipid profiles. "Each value is expressed as Mean ± SEM where n=6. a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to diabetic control". (**A**) HDL level (mg/dl). (**B**) Total cholesterol level (mg/dl). (**C**) Triglyceride level (mg/dl). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

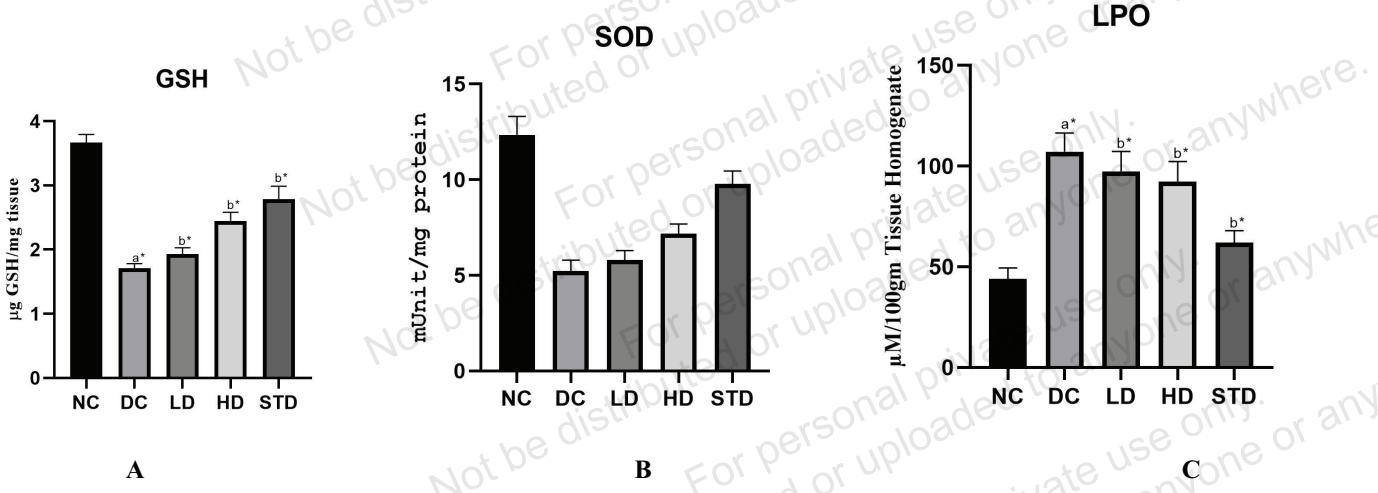


Fig. (6). Estimation of tissue antioxidant parameter. a* $p < 0.05$ when compared to normal control and b* $p < 0.05$ when compared to diabetic control". (**A**) Level of GSH (µg/mg of tissue). (**B**) SOD Activity (mU/mg of protein). (**C**) LPO or MDA content (µM/100 g, tissue homogenate). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

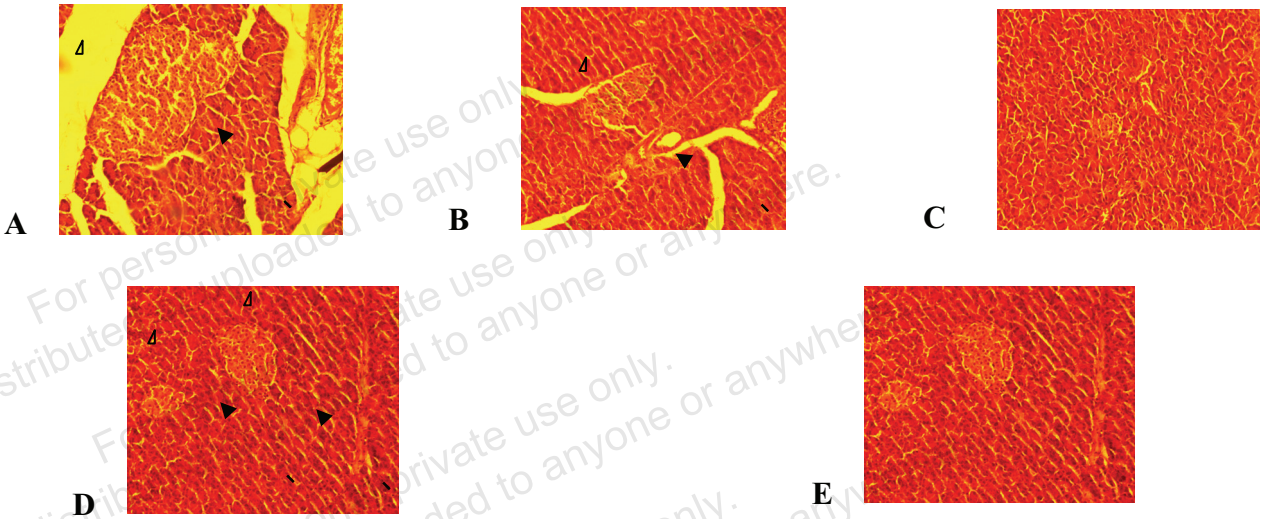
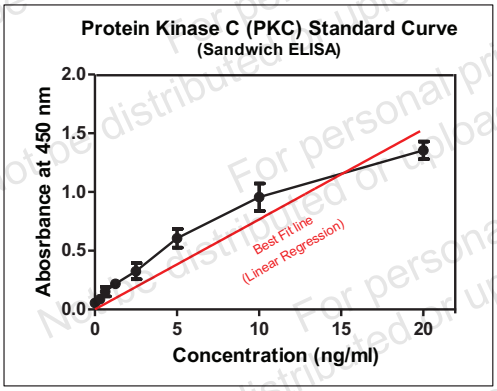


Fig. (7). Histopathology of the pancreas. (**A**) β cell in normal control rats, (**B**) Total destruction of β-cells In STZ control rats, (**C**) Remnants of beta cell in LD treated rats. (**D**) Gradual regeneration of β cells in HD treated rats. (**E**) β cell in normal standard drug treated rats. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).



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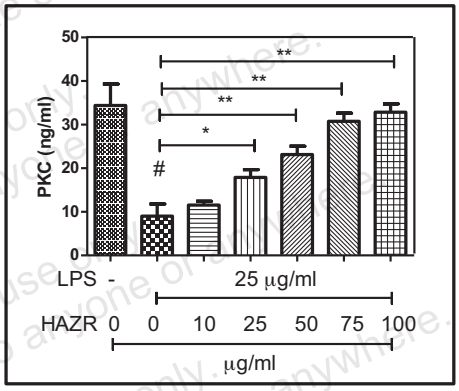

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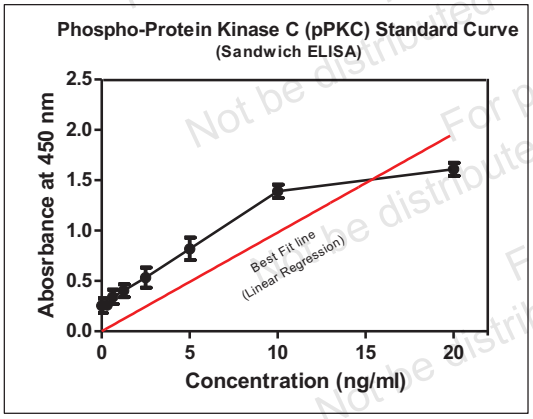

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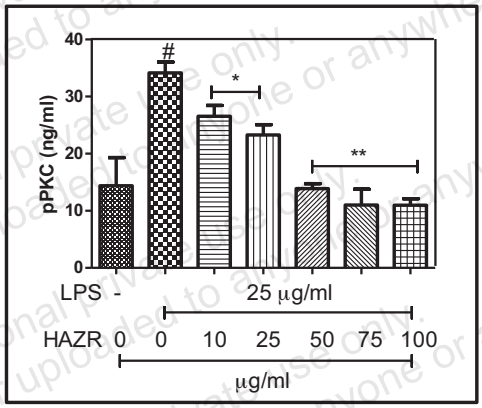

D

Fig. (8). PKC and Phospho- PKC estimation of mouse peritoneal macrophages. Primary macrophages were cultured in RPMI media with 10% FBS in control condition in 5% CO_2 incubator. Treated with or without LPS and different concentration of HAZR followed by estimation of PKC and Phospho- PKC estimation by using commercially available Sandwich ELISA kit. All values are expressed as Mean±SEM (n=3). (**A**) Standard Curve of PKC. (**B**) PKC levels in cells treated with HAZR and LPS. (**C**) Standard Curve of Phospho- PKC. (**D**) Phospho-PKC levels in cells treated with HAZR and LPS. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

The goal of this study was to investigate the traditional belief that the *Zingiber rubens* Roxb. plant has antidiabetic properties. We used an animal model for non-insulin-dependent diabetes mellitus (NIDDM), or type 2 diabetes mellitus in humans, by inducing diabetes in rats through a high-fat diet (HFD) and low doses of STZ [1, 4]. We found that HFD and a low dose of STZ significantly increased the blood glucose level in the experimental animals. However, we observed a dose-dependent antidiabetic effect of HAZR, as shown in the results section. It is well known that diet plays a significant role in the development of diabetes, hypertension, hyperlipidemia, and nephropathy over time. Streptozotocin enters the β-cells *via* a glucose transporter (GLUT2) and causes alkylation of DNA, leading to oxidative stress-mediated damage to pancreatic β-cells [17, 18]. Therefore, the HFD and low-dose STZ-induced experimental animal model is widely used for the evaluation of antidiabetic lead molecules.

Zingiber rubens Roxb. is commonly found throughout the northeast region of India and is known for its antioxidant, antidiabetic, and immunomodulatory properties [6-8]. The root oil of *Z. rubens* contains 24 compounds, mostly monoterpenes (75.3%) with (Z)-citral (30.1%), camphene (9.7%), β-phellandrene (7.5%), and 1,8-cineole (7.0%) being the most dominant. Sesquiterpenoids were also present, with zingiberene (5.3%) being the most significant [8]. The leaves of the plant contain reducing sugar, anthraquinone, terpenoids, flavonoids, saponins, tannins, alkaloids, and cardiac glycosides. A preliminary study showed that the plant's high concentration of flavonoids and phenolic compounds may be responsible for its free radical scavenging activity [8]. We conducted an *in vitro* free radical scavenging assay and obtained significant IC_{50} values, as shown in the results.

In vitro data cannot always predict the effectiveness of a substance *in vivo* [19-21]. We observed moderate inhibitory activity of a plant extract on *in vitro* enzymes but conducted animal experiments to investigate its antidiabetic properties. The extract was found to reduce redox oxidative stress and protect pancreatic beta cells in rats. Monitoring HbA1c levels is important, but lowering them may not always result in a significant decrease in microvascular events associated with diabetes [22, 23].

Several signaling pathways have been found to play crucial roles in the development of diabetes and its complications [24, 25]. In patients with late diagnosis or those trying to manage their blood sugar using oral hypoglycemic agents, the level of Protein Kinase C (PKC) increases, which triggers the activation of TGF-β and its associated signaling mechanisms, leading to diabetic complications [24-27]. A preliminary hypothesis suggests that PKC-mediated TGF-β regulation is responsible for complications like retinopathy, nephropathy, and neuropathy [26, 27]. In order to explore the molecular mechanism of action, we investigated the effect of HAZR on the inhibition of phosphorylation of PKC in mouse peritoneal macrophage cells. Protein kinase C (PKC) plays a crucial role in phosphorylation of AMP-activated protein kinase (AMPK) in endocrine control [28]. HAZR was found to significantly inhibit the phosphorylation of PKC when LPS stimulated the cell. LPS and STZ play an equal inflammatory role in the microenvironment of cells, and therefore, the inhibition of PKC phosphorylation as found in cell culture study may be concluded as the mechanism of oxidative stress-mediated PKC and TGF-β regulation in diabetic individuals [29, 30].

CONCLUSION

The use of traditional medicine has been a global practice for treating various ailments. These medicines contain beneficial bioactive compounds. However, treating chronic diseases can be challenging due to their severe side effects. This compels the need to explore alternatives that can effectively ameliorate the condition and prevent its progression. The present study has shown that HAZR has an antidiabetic effect on diabetic animal models through oxidative stress-mediated PKC and TGF-β regulation in diabetic individuals.

AUTHORS' CONTRIBUTIONS

It is hereby acknowledged that all authors have accepted responsibility for the manuscript's content and consented to its submission. They have meticulously reviewed all results and unanimously approved the final version of the manuscript.

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of Variance
FBG	=	Fasting Blood Glucose
OECD	=	Organisation for Economic Co-operation and Development
OGTT	=	Oral Glucose Tolerance Test
p-NP	=	p-nitrophenol
PKC	=	Protein Kinase C
PMS	=	Phenazine Methosulfate
SALP	=	Serum Alkaline Phosphatase
SGOT	=	Serum Glutamic Oxaloacetic Transaminase

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The animal experimentation protocols at Jadavpur University in Kolkata, West Bengal, India, were thoroughly examined and approved by the Animal Ethical Committee. The University Animal Ethics Committee approved the protocol with the approval number JU/IAEC-22/47 on June 15th, 2023.

HUMAN AND ANIMAL RIGHTS

This study adheres to internationally accepted standards for animal research, following the 3Rs principle. The ARRIVE guidelines were employed for reporting experiments involving live animals, promoting ethical research practices.

RESEARCH INVOLVING PLANTS

An authenticated dealer collected the mature rhizomes/ roots of the plant from the local market. The Botanical Survey of India, Gangtok, Sikkim, Gangtok 737103, India, further authenticated the plant material. The certificate of authentication (SHRC-5/02/2023-24/Tech/272) was securely stored at the School of Natural Product Studies, specifically at the Department of Pharmaceutical Technology, which is located at Jadavpur University in Kolkata, West Bengal, India.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information are available within the article.

FUNDING

The DBT-HBM, India initiative funded the study (BT/PR45281/NER/95/1934/2022, dated 17/03/2022).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

ARRIVE checklist is available on the publisher's website along with the published article.

Supplementary material is available on the publisher's website along with the published article.

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