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Original Article

Phytochemical Profiling by Hyphenated Technique and *Invitro* Enzymatic Anti-Diabetic Activity of Ethanolic Extract of (*Oryza sativa. L Indica*)

M.V. Kumudhavalli[1*], S. Gokul[2], T. Keerthana[1], L. Janarthanan[1], M. Kumar[1].

[1]*Department of Pharmacognosy, Vinayaka Mission's College of Pharmacy, Vinayaka Mission's Research Foundation (Deemed University), 636008 Salem, Tamil Nadu, India*
[2]*Department of Pharmacy, Vinayaka Mission's College of Pharmacy, Vinayaka Mission's Research Foundation (Deemed University), 636008 Salem, Tamil Nadu, India*

**Corresponding Author's Email:* kumudhu27@gmail.com

Abstract

Diabetes mellitus is one of the metabolic disorders caused by insufficient secretion of insulin from pancreas this leads to increase level of blood glucose level. This causes severe side effects like retinopathy, neuropathy and so on. In India the common food is rice (*Oryza sativa*) for their day-to-day life which is having excessive carbohydrate and less fibre content. Hence, there is a chance of increased glycaemic index level in our body. To overcome this problem there is an alternative food black rice (*Oryza sativa. L Indica*) contains more fibre and less carbohydrate when compared to white rice. In this research we focussed the phytochemical analysis and *invitro* pharmacological evaluation by using enzymatic assay method with the use of ethanolic extract of *Oryza sativa. L Indica* by cold maceration process. Phytochemical analysis of extract revealed that presence of various phytoconstituents like flavonoids, saponins, alkaloids, tannins, phenols and glycosides. In Aldose reductase inhibition assay extract showed significant inhibitory property with and IC50 value of 238.7µg/ml. Results of α- glucosidase inhibitory assay showed IC50 value of 383.2µg/ml and IC50 of 228.6 2µg/ml for α- amylase inhibition activity. The anti-diabetic potential may be due to the presence of flavonoids and phenols present in the extract. In these researches it was proved through *invitro* anti-diabetic potential using enzymatic inhibition activity. In future, through *in vivo* method, the same activity will be carried out for further research development towards diabetes. By concluding that black rice can be taken in daily diet by all common people without any complications.

Keywords *: Aldose Reductase, Black Rice Diabetes, α-amylase, α- glucosidase*

Introduction

Diabetes Mellitus (DM)is a category of metabolic syndromes distinguished by high blood sugar volume, abnormal processing of fats, sugars, and proteins, and an increased chance of developing health issues related to blood vessel problems. It can be categorized based on clinical symptoms into type I DM (known as insulin-dependent diabetes mellitus or IDDM) and type IIDM (called non-insulin-dependent diabetes mellitus or NIDDM). The IDF revealed that around 537 million individuals aged 20 to 79 were diagnosed with diabetes in the year 2023 (Ong *et al.,* 2023)**.**The Major five chronic complications of diabetes are hardening of the arteries (atherosclerosis), diabetic foot ulcers, nerve damage (neuropathy), eye damage in people with diabetes (diabetic retinopathy), and kidney damage (nephropathy) (Rorsman & Ashcroft, 2018)**.**

In a good health individual, the regulation of blood sugar levels is maintained by a complex interplay of hormones, in which insulin playing a pivotal role, secreted from pancreas, a tiny biological structure

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positioned near the stomach, it also secretes important enzymes that facilitates breaking down the food. Insulin facilitates the movement of glucose through the bloodstream into muscle, liver, and fat cells, where it serves as a vital source of energy. However, in individuals with diabetes, this delicate balance is disrupted (Venkatesh *et al*., 2021). There are two primary forms of diabetes: Type 1, where the pancreas lacks sufficient insulin production, and Type 2, where the pancreas doesn't make enough insulin or can't process it properly by the body. In both cases, glucose remains in the bloodstream, unable to enter the cells. This situation not only deprives the cells that require glucose for energy but also exposes various organs and tissues to prolonged exposure to high glucose levels, leading to cell damage (Rorsman & Ashcroft, 2018). The typical range for blood sugar is between 80 and 120 mg/dl, or 4 to 6 mmol/l. Levels higher than 200 mg/dl (10 mmol/l) are typically followed by feelings of discomfort and a need to urinate more often, which can result in dehydration. Levels above 300 mg/dl (15 mmol/l) usually demand urgent medical attention and could potentially cause ketoacidosis. (Venkatesh *et al*., 2021). NIDDM caused by association of defective insulin secretion and *insulin resistance* (defective responsiveness of tissues to insulin) (Anderson, 2007).

At this stage, elevated blood sugar levels can be reduced using various techniques and medications that boost the body's reaction to insulin or reduce the sugar produced by the liver. Nonetheless, as the condition advances, the decrease in insulin release becomes more severe, frequently requiring medical insulin injections (Feingold, 2024). White rice (*Oryza sativa*) is a fundamental food in India and is widely eaten in various areas of the southern part of the country, including TN, Kerala, Karnataka, and AP. As white rice is high in carbohydrates, it can raise the glycaemic index in people with diabetes. As a result, replacing, white rice with black rice *Oryza sativa* L*. Indica*– Gramineae (A species of rice)can help control the rise in blood sugar, leading to provide more stable glucose level as it having less glycaemic index and induce satiety(Ardiansyah & Nawawi, 2021).

It is cultivated and grown as branch-like spikes, yielding grains that are the primary economically valuable component of the plant, with the endosperm being the consumable final product. Significantly, black rice is packed with a diverse array of nutrients, including 17 essential amino acids and substantial amounts of antioxidants, in addition to vital vitamins like B and E, proanthocyanidins, carotenoids, anthocyanins, flavanols, phytic acid, and phenolic acid. Furthermore, it offers a good amount of dietary fibre, iron, zinc, and phosphorous, rendering it an extremely beneficial component of a diet (Hiei, Ishida & Komari, 2015). People currently facing diabetes, obesity and other health disorders can be compensate with black rice as their routine meal. As this crop contains flavonoids it may have anti-diabetic activity and anti-oxidant property, the focus on specific enzymatic anti-diabetic activity, and the potential to link phytochemicals directly to therapeutic outcomes, all of which may open new doors for diabetes treatment and the broader use of rice in medicine. hence this research focus to evaluate its potency towards the Type-II diabetes.

Material and Methods

Plant materials and chemicals

Oryza sativa L. Indica was obtain from local market in Salem. The reagents, chemicals, and materials utilized in this research are analytical grade were purchased from Sigma-Aldrich.

Instruments: GC-MS (Perkin–Elmer Gc-Clarus 500).

Extraction Procedure

O. sativa L.Idica was bought, washed and dried in the shade. Following that, it was pulverized into powder. This powdered material was soaked in ethanol using the cold percolation process for a period of 72 hours. Afterwards, it was filtered and concentrated. The crude extract was stored in a well closed container for further evaluation (Sankeshwari *et al.,* 2018).

Phytochemical Analysis

Phytochemical analysis was carried out for the crude ethanolic extract of *O. sativa L. Indica* to determine the existing of alkaloids, Saponins, tannins, phytosterol, flavonoids, glycosides, and phenols, triterpenoids, anthraquinones. (Jana & Shekhawat, 2010; Prakash & Jain, 2011).

GC-MS analysis of Oryza sativa L. Indica (Perkin–Elmer GC-Clarus 500)

Electron ionization system was operated with an ionization energy of 70 eV. Pure helium, with a purity of 99.999%, was utilized at a steady rate of 1 ml per minute. An injection volume of 2 microliters was utilized, with a split ratio of 10:1, isothermal temperature was maintained. A mass spectrum was obtained at a potential energy of 70 eV, using a scan duration of 0.5 seconds, and fragments with 45 to 450 Da in mass. It was run for 35 mins (Devarasu *et al.,* 2024).

***INVITRO* STUDIES**

Buffer Preparation

Phosphate buffer (80mM) pH 7.0

12.48 g of Sodium dihydrogen phosphate was dissolved in 1000 ml of de-ionised H_2O and kept as solution-A. Disodium hydrogen phosphate anhydrous (11.35 g) were dissolved in 1000 ml of de-ionised H_2O and kept as solution-B. Solution A (39 ml) with solution B(61 ml) was mixed and made up to 200 ml with de-ionised H_2O.

The care and procedures for handling animals were conducted in accordance with the guidelines set by the Institutional Animal Ethical Committee. (12/IAEC/MG/04/2023-I).

Aldose reductase preparation from Rat

Raw Aldose reductase was extracted from the lenses of rats. The eyes of male rats, aged 9 weeks, were surgically removed. The methods for the treatment and management of animals adhered to all standards and were granted authorization by the Institutional Animal Ethics Committee. The lenses were cut from the back and ground into a fine paste using a homogenizer, with a total volume of 10 Liters and a potassium phosphate buffer solution. This mixture was then spun in a centrifuge at a speed of 15,000 g for a duration of 30 minutes at a temperature of 4°C. The liquid that remained after this process was collected and used as the Aldose reductase source.(Ramkumar *et al.,* 2020; Lavanya et al., 2020).

Assay Procedure

The test solution in 1 mL had a concentration of 50 μM potassium phosphate, along with lithium sulphate 0.4 mM, 2-mercaptoethanol 5 μM, DL-glyceraldehyde 10 μM, NADPH 0.1 mM, and a mixture of enzymes from rat lenses. Corrective blanks were used as needed. The test solution was kept at 37ºC and started by adding NADPH at the same temperature (Arathy *et al.,* 2020). The alteration in the transmission at 340 nm resulting from the oxidation of NADPH was quantified using spectrophotometry.

α – Glucosidase Preparation

Rat's intestine was taken away and stored in an ice-cold solution of 80 mM phosphate buffer, maintaining a pH of 7.0. The intestine is cut open, with its inner lining scraped off with a piece of glass rod and thoroughly mixed using a homogenizer, which is four parts of buffer to one part of the intestine's tissue (v/w). Throughout this process, the tube is kept chilled with crushed ice. To remove any unwanted debris like nuclei and large cell fragments, the mixture is subjected to centrifugation at speeds between 2000 to 4000 rpm for a duration of 10 minutes. The liquid above the sediment is then stored at – 20°C. According to Lowry's method, the approximate protein content of the mixture is around 0.5 grams per decilitre. The enzyme of interest is made using phosphate buffer (Arathy *et al.,* 2020).

Assay Procedure

Combine the enzyme (50 μL) with buffer (250 μL) and kept at 37°C for half an hour. Then it was

poured in 500 µL of a sugar solution and kept at same temperature for another 20 minutes. To stop the reaction, it was heated in a boiling water bath for 2 minutes, then cooled. Amount of glucose by using the Glucose Oxidase method was determined. 100 µL of the extract was mixed with 500 µL of the glucose reagent (Glucose reagent kit) and kept at moderate temperature for 10 minutes.(Ramkumar *et al.,* 2020; Lavanya et al., 2020). Absorbance was at 510 nm.

α - Amylase Inhibition Assay

Pancreatic α-amylase hydrolyses the 2-chloro-4- nitrophenol α-D – maltotrioside (CNPG3) to release 2-chloro-4-nitrophenol and form 2-chloro-4- nitrophenol α-D – maltoside (CNPG2), malt triose and glucose. The rate of formation of the 2-chloro-4-nitrophenol can be measured at 405nm to give direct measurement of amylase in the sample.

Procedure

Mixing 50 µL of enzyme (isolated from porcine pancreas), 120 µL of phosphate buffer, and the sample (with a pH of 7.0) together and the mixture was kept at 37°C for ten mins. Next, 250 µL reagent (CNPG3) was added and left at the same temp for 8 mins. To stop the reaction, the solution was heated in water bath for two mins, and then cool it and transmittance evaluated at 405 nm.

Results and Discussion

The extract was obtained from cold maceration method. The extract obtained showed brown colour with viscous consistence. The ethanolic extract yields about 25%. It was soluble in ethanol, DMSO, chloroform and water.

Figure 1: Oryza sativa l. Indica

Figure 2: Cold Maceration of Oryza sativa l. Indica

Phytochemical Screening

The ethanolic extract of *Oryza sativa L. Indica* was subjected to phytochemical screening, the results revealed the existence of phytoconstituents like alkaloids, flavonoids, glycosides, phytosterol, phenols, saponins, and tannins were present these Phytoconstituents are believed to be responsible for therapeutic effect, hence the extract was further subjected to various in -vitro enzymatic activity to prove anti-diabetic effect.

Table 1: *Phytochemical screening of ethanolic extract of Oryza sativa L. Indica*

SL.NO	TESTS	OBSERVATION
1	TANNINS	+
2	TRITERPENOIDS	-
3	ALKALOIDS	+
4	PHENOLS	+
5	PHYTOSTEROL	+
6	FLAVONOIDS	+
7	GLYCOSIDES	+
8	ANTHRAQUINONES	-
9	SAPONINS	+

GC-MS analysis of ethanolic extract of *Oryza sativa L. Indica*

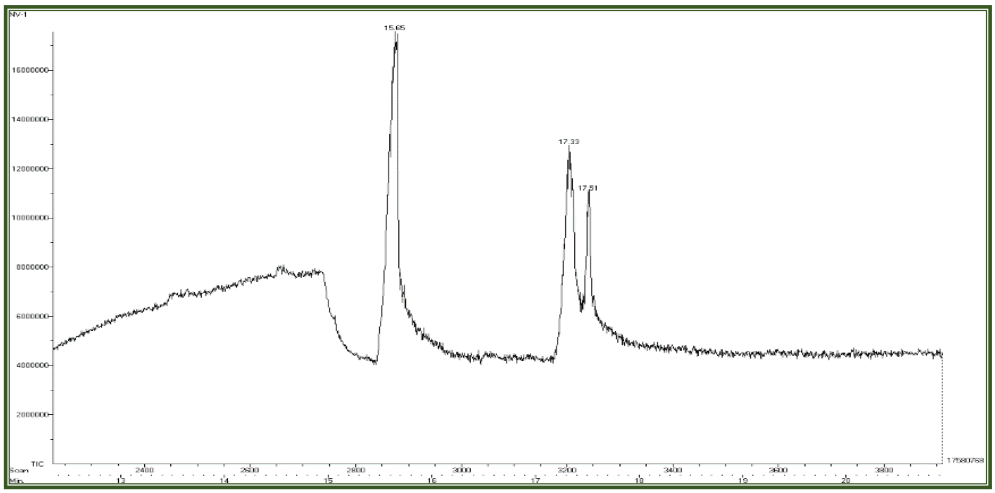

Figure 3: *Chromatogram of ethanolic extract Oryza sativa L. Indica*

The chromatogram shows the various peaks which corresponds to various phytoconstituents like cyclohexanol and nonadecene

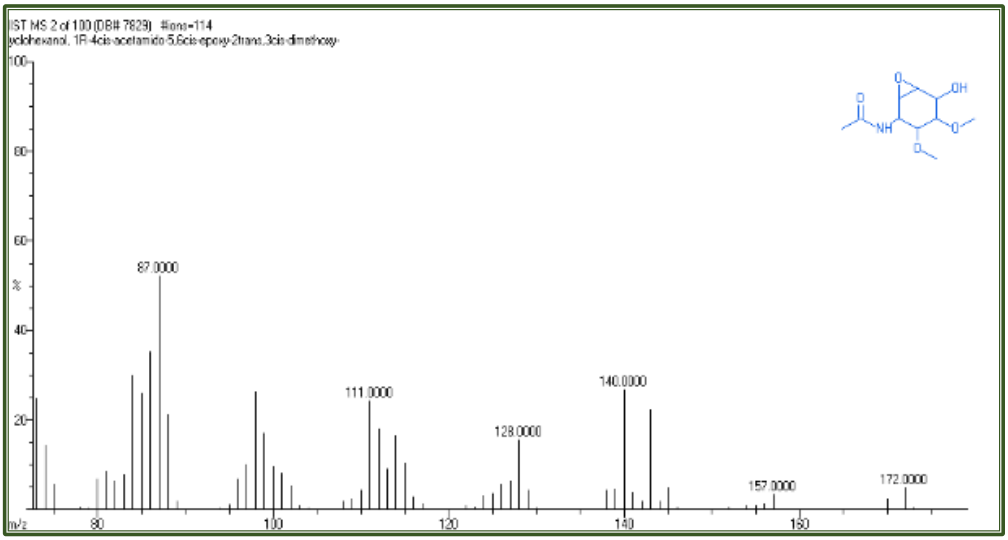

Figure 4: *Structural confirmation of cyclohexanol*

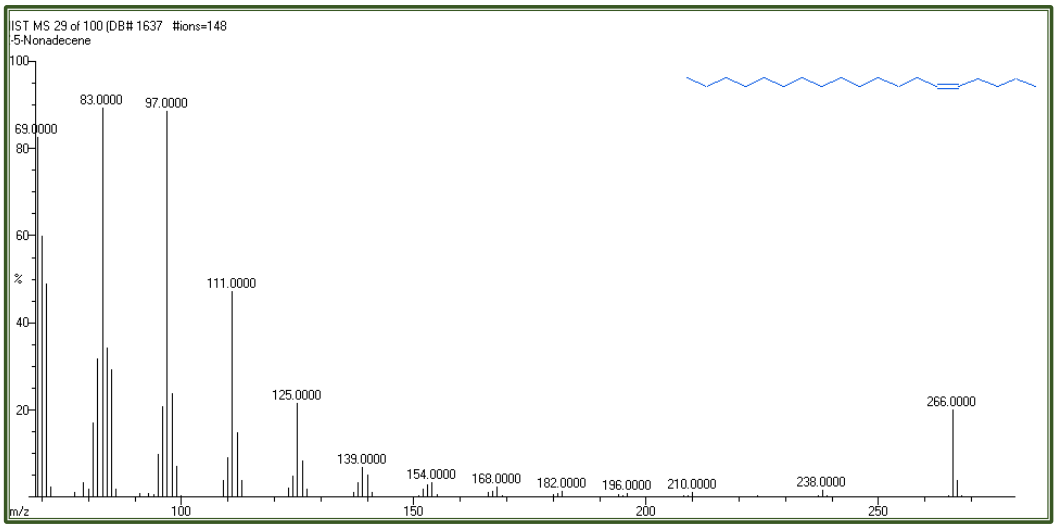

Figure 5: *Structural confirmation of nonadecene*

The chromatogram generated by GC-MS displays peaks at specific retention times, indicating the presence of different compounds.

INVITRO Anti-Diabetic Activity

This study investigated by evaluating *Oryza sativa L. Indica* inhibitory activity against crucial enzymes associated with diabetes, including Aldose reductase, α -glucosidase. The antidiabetic potential of this extract is presented in Tables No. 1, 2, and 3 along with their respective IC50 values.

Aldose Reductase Inhibition Assay

Table 2: *Aldose Reductase Inhibition Assay of Ethanolic extract of Oryza sativa L. Indica*

GROUPS	CONC (MG/ML)	OD= 405NM	% INHIBITION	IC50 MG/ML
CONTROL	0	0.659	0.00	0
ACARBOSE	0.3125	0.524	22.02	4.0
	0.635	0.467	32.69	
	1.15	0.343	42.25	
	2.4	0.289	53.31	
	6	0.179	71.74	
	10	0.119	82.71	
ETHANOL EXTRACT	50	0.516	12.02	238.7
	100	0.415	35.09	
	200	0.512	22.53	
	400	0.515	23.07	
	800	0.436	36.20	
	1600	0.321	41.74	

This research indicates that the sample exhibits significant inhibitory properties against aldose reductase, as evidenced by its IC50 value of 238.7µg/ml. When compared to the standard acarbose with an IC50 value of 4µg/ml, these findings imply that this method could be promising in the prevention of diabetes.

α-Glucosidase Inhibition assay:

Table 3: *α-Glucosidase Inhibition assay of Ethanolic extract of Oryza sativa L. Indica*

GROUPS	CONC. MG/ML	OD = 405 NM	% INHIBITION	IC50 µG/ML
CONTROL	0	0.865	0.00	0.00
ACARBOSE	0.3235	0.865	12.24	
	0.654	0.743	23.90	
	1.32	0.654	31.24	3.878
	2.6	0.523	44.20	
	5	0.454	51.31	
	10	0.186	81.21	
ETHANOL EXTRACT	50	0.875	31.27	
	100	0.954	35.18	
	200	0.850	29.73	383.2
	400	0.592	38.71	
	800	0.954	56.04	
	1600	0.869	59.98	

The results of the α- glucosidase inhibitory assay indicated that this extract had inhibitory activity against this enzyme, with an IC50 value of 383.2µg/ml. the standard drug Acarbose exhibited a much lower IC50 value of 3.8µg/ml, indicating its superior potency.

α- Amylase Inhibitory Activity

Table 3: α- Amylase Inhibitory assay of Ethanolic extract of *Oryza sativa L. Indica*

GROUPS	CONC. µG/ML	OD = 590 NM	% INHIBITION	IC50 µG/ML
CONTROL	0	0.81	0.00	0
ACARBOSE	0.3135	0.49	8.97	2.32
	0.615	0.48	17.92	
	1.35	0.40	31.58	
	2.4	0.24	36.64	
	4	0.28	54.90	
	10	0.37	67.08	
ETHANOL EXTRACT	50	0.84	15.36	228.6
	100	0.91	33.54	
	200	0.76	51.18	
	400	0.61	63.34	
	800	0.50	63.83	
	1600	0.31	74.54	

The results of the α- amylase inhibitory assay indicated that this extract had inhibitory activity against this enzyme, with an IC50 value of 228.6µg/ml. Acarbose, the standard drug, exhibited a much lower IC50 value of 2.32µg/ml, indicating its superior potency.

Discussion

Nowadays black rice was commonly used by diabetic, obesity and also by common healthy people to enhance their health. As diabetes is a chronic condition, in this research an attempt was taken to evaluate the potency of black rice in diabetes by invitro method using the enzyme aldose reductase and α-glucosidase (Das *et al.,* 2023) Fig.1 Black rice used for the study, which was mainly grown in the northern and southern parts of India. It grown as crop over 1m tall and also 5m long in deep water. The ripened rice grain contains more beneficial nutritious value and bioactive constituents.

Ethanolic extract was obtained by cold maceration process, phytochemicals present in the extract was determined by various phytochemical assay (Singh, Kapoor & Bhatnagar, 2021). From the phytochemical assay results (Table No. 1) it was confirmed the presence of flavonoid and phenols which may be responsible for the anti-diabetic activity. In GC-MS analysis the chromatogram was obtained (Fig.no. 3). The peaks at 15.65 and 17.33 (Graph-1) correspond to cyclohexanol and nonadecene (Fig.no.4 & 5) respectively. GC-MS analysis confirmed the presence of cyclohexanol and nonadecene in the mixture based on their characteristic retention times (Sales *et al.,* 2012).

The findings from aldose reductase assay (Table 2) suggested that extract may have potential property preventing diabetic complications such as retinopathy and nephropathy by inhibiting Aldose reductase activity. While extract showed its potency is considerably lower compared to the standard drug acarbose. Alpha amylase is an enzyme responsible for the breakdown of starch into glucose in the digestive system. Inhibition of this enzyme can help control post-meal blood sugar spikes (Bhatia, Singh & Arora, 2019). The results of the alpha amylase inhibitory assay indicated that the extract may be considered as a potential starch blocker, slowing the absorption of starch into the body, and thereby potentially helping to control postprandial blood glucose levels. The presence of polyphenols and flavonoids in the extract is suggested as a contributing factor to its alpha amylase inhibitory activity. The inhibition of alpha glucosidase by the ethanolic extract suggests that it may delay the degradation of

carbohydrates, leading to reduced glucose absorption and potentially lowering postprandial blood glucose levels (Ashokkumar *et al.,* 2020).

Conclusion

The study has established black rice as a good alternative to white rice in the strategy for reducing the risk of diabetes due to the low glycaemic index with high fibre content. During phytochemical screening, certain beneficial compounds such as flavonoids and phenolic compounds were found which may contribute to its anti-diabetic actions. The ethanolic extract exhibited good in vitro anti-diabetic action: aldose reductase inhibition with an IC50 value of 238.7 µg/ml and alpha-glucosidase inhibition with an IC50 value of 328.2 µg/ml, higher than that of the standard drug acarbose. The key shortcoming of this study is that it was conducted *in vitro*, and therefore may or may not have a good relation to in vivo actions and effectiveness. Moreover, this focus on a single method of extraction and certain compounds can have limited generalizing in results. The need for further studies using advanced analytical techniques for structural elucidation and molecular aspects of the identified compounds, along with *in vivo* studies to establish the therapeutic potential of black rice in the management of diabetes, still persists. Various extraction methods and processing techniques should be investigated to further develop its bioactive properties.

Conflict of Interest

The authors declare that they have no competing interest.

Acknowledgment

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