



## **Antidiabetic Effect of *Katakakhadiradi kashayam* by Improving the Insulin Expression and Glucose Metabolising Enzyme**

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### **Authors' contributions**

This work was carried out in collaboration among all authors. Author AJS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MK and MR managed the analyses of the study. Authors KP and MRKR managed the literature searches. All authors read and approved the final manuscript.

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### **ABSTRACT**

Many plants provide a rich source of bioactive chemicals, which are free from undesirable side effects and possess powerful pharmacological actions. The present study was carried out to find the antidiabetic effect of *Katakakhadiradi kashayam* (KKK) by improving the insulin expression and regulating properly the glucose metabolising enzymes. The diabetes was induced in combination with streptozotocin and nicotinamide injection to Wistar rats. Diabetic rats were treated

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with *Katakakhadiradi kashayam* orally at doses of 100, 200 and 300 mg/kg/bw for 28 days, and the obtained results of parameters were compared with glibenclamide. The antidiabetic effect of *Kashayam* was measured by the expression of insulin by immunohistochemistry and restoring the normal clinical values of glucose metabolizing enzymes. The present study specified that hyperglycemia leads to pathological conditions in pancreatic tissue with decreased expression of insulin in  $\beta$ -cells whereas the *Katakakhadiradi kashayam* normalised the production of insulin. The study found that the antihyperglycemic activity of *Katakakhadiradi kashayam* L. is mainly due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output and maintaining the glucose metabolising enzymes.

**Keywords:** Diabetes; hyperglycemia; insulin; immunohistochemistry; *Katakakhadiradi kashayam*.

## 1. BACKGROUND

Diabetes is a complex metabolic disease that occurs due to inflammation in immunological process. Insulin resistance due to insulin signalling inhibition results in a series of immune responses that exacerbate the inflammatory state, which leads to hyperglycemia. A better comprehension of how immune dysfunctions happen during hyperglycemia can lead to unique treatments and preventions for infectious diseases and T2D comorbidities, therefore improving the outcome of infectious disease treatment in T2D patients [1].

The detection of insulin in islets of  $\beta$ -cells of pancreas gives clear idea about the glucose conditions in blood. The glucose metabolism critically regulates insulin secretion and metabolic functions of various cells [2]. Depending on cell types, rates of glycolysis are determined at various steps of glycolysis that are subjected to the control of key metabolic and regulatory enzymes, which comprise glucose phosphatase, fructose-1,6-bisphosphatase and hexokinase. These enzymes are controlled by both hormonal and nutritional signals at the levels of post-translational, translation, and transcription modifications. Glycolysis is involved in the control of hepatic glucose production in the hepatocytes; the excessive alteration in glucose metabolism contributes to hyperglycemia in diabetes [3].

For decades, many plants have been an important source of effective antidiabetic drugs. In developing countries, especially, herbs are used for treating diabetes to overcome the burden of the cost of conventional medicines to the population [3]. Hence, herbal drug therapy has an effect on protecting  $\beta$ -cells and smoothing out the variations in glucose levels. Generally, there is very little biological knowledge on the specific modes of action in diabetes treatment,

but most of the plants present in *Katakakhadiradi kashayam* (KKK) have been found to have substances like glycosides, terpenoids, alkaloids, flavonoids etc. that are frequently implicated as having antidiabetic effects [4]. Almost all the 12 components of *Katakakhadiradi kashayam* formulation have scientific evidence to have beneficial role against diabetes. Studies showed that medicinal plants of *Katakakhadiradi kashayam* have experimental evidence of anti hyperglycemic activity [5]. This *kashayam* has been in wide clinical practice by the Ayurvedic physicians in diabetic like clinical condition having good therapeutic result [6]. Therefore, it is appropriate to suggest that the components herbs of this *kashayam* acts in a complimentary and/or synergistic mode to pronounce the antihyperglycemic effect of the components in the whole formulation [7]. The aim of this immunohistochemical study was to evaluate pancreas insulin protein expression in relation to glucose metabolizing enzymes in diabetes. The present study was designed to investigate the antidiabetic effect of *Katakakhadiradi kashayam* by improving the insulin expression and glucose metabolising enzyme.

## 2. MATERIALS AND METHODS

### 2.1 Plant materials and Formulation

All the plant materials were collected from the herbal garden and drug store of Ayurveda college, Coimbatore, Tamil Nadu, India. *Katakakhadiradi kashayam* is a herbal decoction prepared from 10 grams each of the following ingredient plants: *Strychnos potatorum*, *Acacia catechu*, *Emblica officinalis*, *Berberis aristata*, *Biophytum sensitivum*, *Barringtonia acutangula*, *Cyperus rotundus*, *Salacia reticulata*, *Curcuma longa*, *Terminalia chebula*, *Mangifera indica*, *Cassia mimosoides*.

## 2.2 Animals

Adult male albino Wistar rats (6 weeks), weighing 150 to 200 g were used for the present antidiabetic study. The rats were kept in clean polypropylene cages and maintained in a well-ventilated temperature regulated animal cage with a continuous 12 h light/dark schedule. The rats were given standard rat pelleted diet and filtered drinking water was given *ad libitum*.

## 2.3 Experimental Induction of Diabetes

The rats were kept for overnight fasting. Blood samples were collected from the tail tip in EDTA-containing microtubes and checked the primary fasting blood glucose. Streptozotocin and nicotinamide were dissolved in citrate buffer (pH 4.5) and normal saline respectively. Streptozotocin (60 mg/kg) was dissolved in citrate buffer (pH 4.5) and intraperitoneally injected to the overnight fasted rats, after 15 minutes 120 mg/kg of nicotinamide was administered intraperitoneally to induce non-insulin dependent diabetes mellitus. Hyperglycemia was observed after 72 hours by the increased blood glucose levels. The rats with blood glucose level higher than 250 mg/dl were used for the present study [8].

## 2.4 Study Design

The rats were classified into 6 groups each having six animals. Group I rats received normal saline 1 ml/kg bw. orally; Group II rats injected with streptozotocin 60 mg/kg bw. and nicotinamide 120 mg/kg intraperitoneally to induced diabetes; Group III diabetic rats treated with Glibenclamide 20 mg/kg orally for 28 days; Group IV diabetic rats treated with *Katakakhadiradi kashayam* 100 mg/kg orally for 28 days; Group V diabetic rats treated with KKK 200 mg/kg orally for 28 days; Group 6 diabetic rats treated with *Katakakhadiradi kashayam* (KKK) 300 mg/kg orally for 28 days.

Animals were anesthetized via the respiratory route by exposing them to ether for approximately 2 minutes in a transparent acrylic jar and after which the pancreas was dissected out. Care was taken not to damage the pancreatic surface.

## 2.5 Assay of Glucose-6-phosphatase

The tissue homogenate 0.06ml was pipetted into an assay mixture containing 26.5 mM glucose, 1.8 mM Ethylenediaminetetraacetic acid (EDTA), both previously adjusted to pH 6.5, 2 mM

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>), 0.5-0.7 IU/ml mutarotase, 5-7 UI / ml glucose dehydrogenase, and 100 mM imidazole-HCl, pH 6.5 (final concentrations). The mixture was preincubated for 4 min before monitoring the increase in absorbance (Nicotinamide adenine dinucleotide (NADH) production) per time unit for an additional period of 3.5 min. One unit of glucose-6-phosphatase activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 picomoles per litre (pmol) of glucose-6-P per minute under the specified conditions.

## 2.6 Assay of Fructose 1,6 Bisphosphatase

The assay medium in a final volume of 2ml contained 1.2ml of buffer, 0.1ml of substrate solution, 0.25ml of MgCl<sub>2</sub>, 0.1 ml of KCl solution, 0.25ml of EDTA solution and 0.1ml of enzyme. The incubation was carried out at 35°C for 15min. The reaction was terminated by the addition of 1ml of Tricarboxylic acid cycle (TCA). The suspension was centrifuged, and the phosphorus component of the supernatant was found by the method of Fiske and Subbarow (1925). Protein estimation was done by the method of Lowry *et al.* (1951). The activity of the enzyme was expressed as nmoles of inorganic phosphate (Pi) liberated/min/mg protein.

## 2.7 Assay of Hexokinase

The incubation mixture containing 2.5ml buffer, 1ml of substrate, 0.5ml ATP, 0.1ml each of MgCl<sub>2</sub> and sodium fluoride and 0.5ml each of KH<sub>2</sub>PO<sub>4</sub> and KCl was preincubated at 37°C for 5min. The reaction was initiated by the addition of 2ml of enzyme extract. About 1ml of aliquot of the reaction mixture was removed immediately (zero time) and added to tubes containing 1ml of 10% Tricarboxylic acid cycle (TCA). After 30 min incubation, 1.0 ml of aliquot of the above reaction mixture was added to a separate set of tubes and the reaction was stopped by the addition of 1.0ml of TCA. After the samples were precipitated and centrifuged, the supernatants were used for the estimation of glucose by ortho toluidine method of Vidal *et al.* (2019). The enzyme activity is expressed as n moles of glucose utilized for the formation of glucose-6-phosphate/min/mg protein.

## 2.8 Immunostaining of Insulin

Immunolocalization of insulin in cells was done by 'indirect peroxidase' procedure. Hsu and Rain

(1981), incorporating the modifications of Slot *et al.* (1991) method. Freshly dissected tissue (less than 3 mm thick) was fixed at 37°C using formalin (10%) for 24-48 hour. The tissue was washed with running water. The tissue was dehydrated through 70%, 80%, and 95% alcohol, 45 min each, followed by 3 changes of 100% alcohol, 1 hour each. The tissue was cleared through 2 concentrations of xylene, 1 hour each. The tissue was immersed in paraffin, for 1 hour. Then the tissues were embedded in paraffin block (cooled at -5°C). The paraffin-embedded tissue block sectioned at a thickness of 5-8 µm on a microtome and was floated in water bath at 40°C. The sections were transferred onto glass slides. The slides were left to dry whole night and stored at room temperature. For this purpose, an HRP/DAB detection immunohistochemistry (IHC) kit was used conferring to the manufacturer's protocol. The sections were dehydrated over alcohol series after the paraffin got eliminated in xylene. Incubating in hydrogen peroxide (3%) (30 min at RT) leads to the initiation of endogenous peroxidase action and was arrested. By incubating using experimentally prepared normal goat serum (30 min), the non-specific binding sites were arrested.

The section was then immune-reacted with 10 µg/mL primary antibodies against insulin overnight at 4°C, respectively after antigen retrieval (100 × Citrate Buffer) (20 min) in a pressure cooker and arresting non-specific binding sites using protein block. The section was submerged in phosphate-buffered saline (PBS) for negative control. The section was then kept for 60 min with anti-Ib 1:250, anti II 1:100 and anti IIIa 1:100 at room temperature. The section was kept along with biotinylated anti serum at RT for 60 min after a rinse with PBS. The sections were then kept in the working streptavidin HRP solution at RT (60 min) and rinsed in three changes of PBS. At last, the section was kept for 30 min with DAB-hydrogen peroxide and rinsed in water (H<sub>2</sub>O), the sections were counterstained and observed using a light microscope.

## 2.9 Statistical Analysis

The mean value and standard error were measured for each parameter. The analysis was carried out by using Sigma Plot 13 (Systat software USA). Whenever significant differences were observed, mean values were compared using one way ANOVA. A probability of less than 0.05 was cogitated significant.

## 3. RESULTS AND DISCUSSION

Despite the great strides that have been made in the understanding and management of diabetes, the disease and disease related complications are increasing unabated despite presence of known antidiabetic medicine in the pharmaceutical market. Ayurveda offers a significant optimism through its multi-herb formulations used under ayurvedic principles to manage this multi-factorial menace challenging the global population [9,10].

Boost of intracellular free glucose metabolic enzymes and its metabolites in the glomeruli is related to preserving kidney function in both T1DM and T2DM. The renal profile of increased glycolytic enzymes and decreased toxic glucose metabolites is reflected in the circulation, assisting their use as biomarkers for endogenous renal protective aspects in individuals with diabetes [11].

Glucose 6 phosphatase activities were higher in streptozotocin and nicotinamide induced diabetic rats when compared with normal control rats. Glibenclamide treatment to the diabetic rats caused a fall in G-6-Pase concentration similar to control group. KKK (300mg) treatment produced decrease in the concentration of G-6-Pase when compared to the standard group, but lower than control group. None of the treatment could return the G-6-Pase levels back to normal values (Fig. 1).

F6 Pase activities were increased in diabetic rats when compared to control rats. Glibenclamide treated rats showed significant decrease in F6Pase when compared to diabetic rats. KKK at a dose of 100 and 300 mg/kg has been found to decrease F6Pase when compared to diabetic rats (Fig. 3).

Fructose 1,6-bisphosphatase (FBPase) , a key enzyme in gluconeogenesis and a potential drug target in T2DM treatment. The protein is also related with a rare inherited metabolic disorder and some cancer cells lack FBPase effect which promotes glycolysis facilitating the Warburg effect [12]. G6PD is the vital source of the major intracellular reductant, NADPH, which is required by many enzymes, comprising enzymes of the antioxidant pathway. High glucose damages G6PD effect and elevates ROS in β cells. Specific inhibition of G6PD alone results in reduced β-cell survival and diminished β-cell proliferation. The hypersecretion of G6PD prevents the high-glucose-mediated increase in

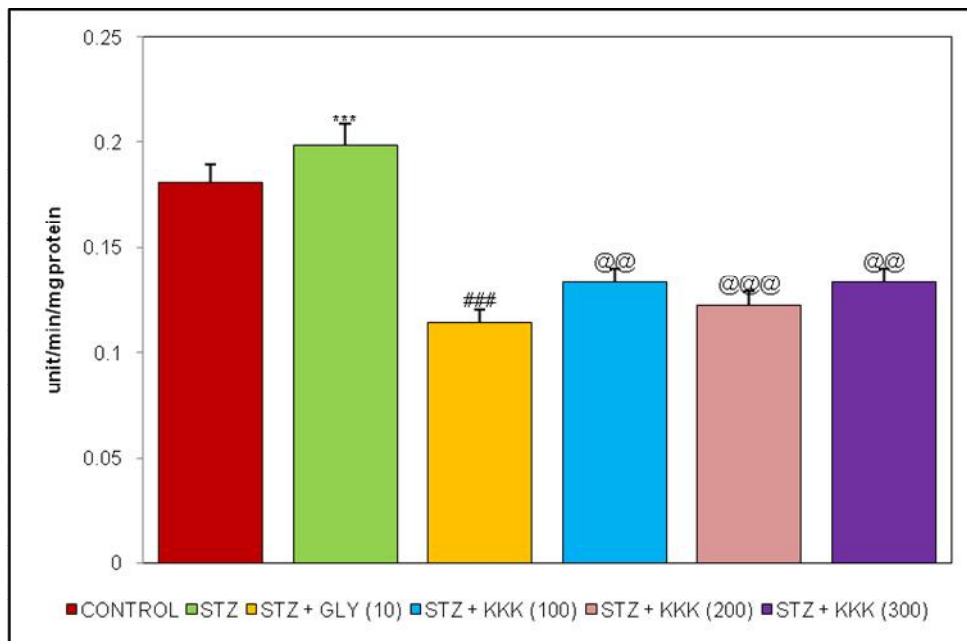
ROS. Taken together, these data support a significant role for decreased G6PD playing a central role in the high-glucose-mediated increase in ROS, which leads to  $\beta$ -cell dysfunction and death [13]. This generates abnormal elevation in glycolytic intermediates, driving cell dysfunction, glycolytic overload, vulnerability to the injuring actions of hyperglycemia in diabetes, explaining tissue-specific pathogenesis [14].

In the present study, insulin immunochemical stain of pancreas showed that normal control group has strong immune reactivity of insulin in beta cells that occupies most of the pancreatic islet. Diabetic rats showed marked decrease in beta cell population and significant reduction immunohistochemical expression of insulin. Glibenclamide and KKK (200 and 300mg) treated group showed normal immunohistochemical expression of insulin in the  $\beta$  cells compared to KKK (100mg) showed shows normal immunoreactivity in  $\beta$  cells of the pancreatic islets (Fig. 2).

Diabetic group showed damage in the cells because of mild deterioration in the pancreatic

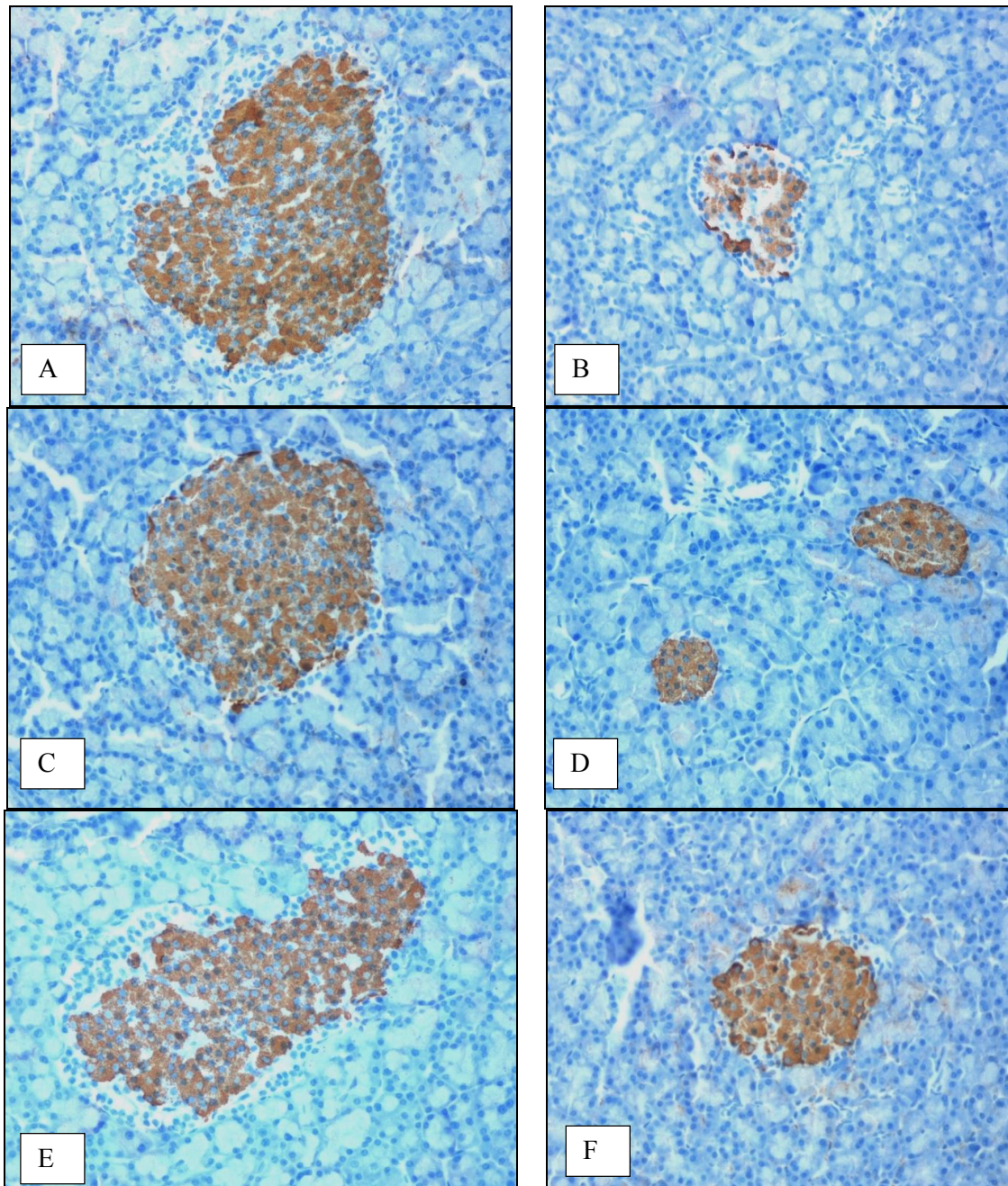
nucleus and  $\beta$ -cells. This occurred due to conditions of hyperglycemia causes pancreatic cells to diminish insulin function [15]. Furthermore, the hyperglycemic condition can also cause oxidative stress, because of increased free radical generation. Free radicals can attack surrounding body cells, including cells in pancreatic that were having damaged [16,17]. The immunohistochemical expression of insulin in pancreas in streptozotocin and nicotinamide combination induced diabetes and its treatment with this herbal formulation, *Katakakhadiradi kashayam* as not yet described till now.

Recently, *Katakakhadiradi kashayam* have been prescribed by ayurvedic doctors for diabetic patients globally and have been used for digestive and antihyperlipidemic remedies [18]. Antihyperglycemic activity of *Katakakhadiradi kashayam* is mainly due to their potential to re-establish the effect of pancreatic tissues by making an increase in insulin output or hinder the intestinal absorption of glucose or to the metabolite facilitation in insulin dependent processes [19,20].

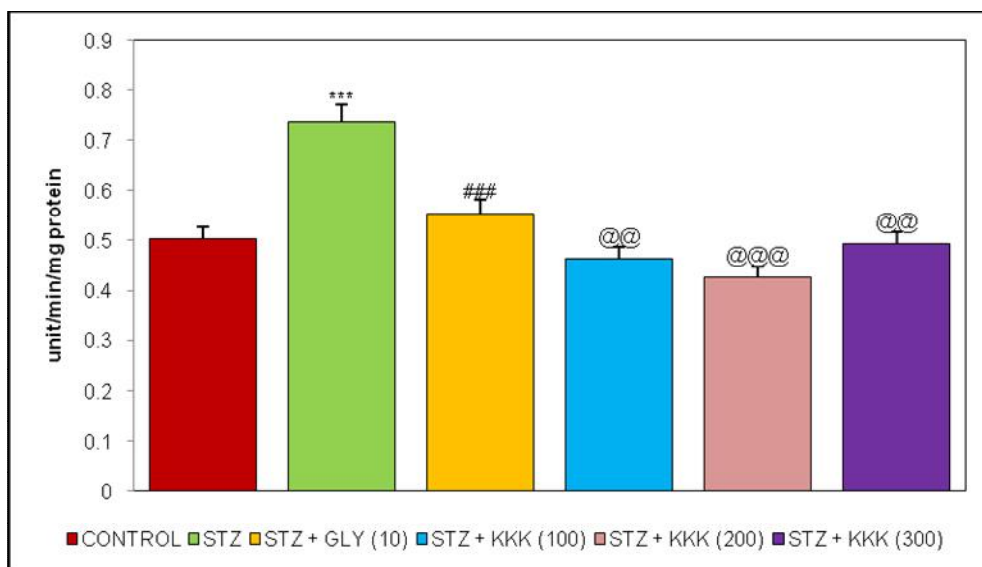


**Fig. 1. Effect of *Katakakhadiradi kashayam* on glucose 6 phosphatase enzymes in streptozotocin induced diabetic rats. Values are expressed as mean  $\pm$  SEM (n=6); \*\*\*p<0.01 significant as compared with control group; ###p<0.05, @@p<0.01, @@@p<0.001 significant as compared with diabetic group**





**Fig. 2. Expression of insulin in pancreas using Immunohistochemistry (100x). A: Group 1: Normal control group, B: Group 2: Diabetic group, C: Group 3- Diabetic + Glibenclamide, D: Group 4- Diabetic + KKK (100 mg), E: Group 5- Diabetic + KKK (200 mg), F: Group 6- Diabetic + KKK (300 mg). Fig. 1. Insulin immunochemical stain of pancreas: A: Control (40x), B: STZ (40x), C: STZ+GLY (10) (40x), D: STZ+ KKK (100) (40x), E: STZ+ KKK (200) (40x), F: STZ+ KKK (300) (40x)**



**Fig. 3. Effect of *Katakakhadiradi kashayam* on fructose 1,6 bis phosphatase enzyme levels in streptozotocin induced diabetic rats. Values are expressed as mean  $\pm$  SEM (n=6); ns-non significant, \*\*\*p<0.01 significant as compared with control group; ###p<0.05, @p<0.01, @@@p<0.001 significant as compared with diabetic group**

#### 4. CONCLUSION

The study found that the antihyperglycemic activity of *Katakakhadiradi kashayam* is mainly due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output and maintaining the glucose metabolising enzymes

#### CONSENT

It is not applicable.

#### ETHICAL APPROVAL

All animal experiments were conducted after getting approval from the ethical committee and in accordance with the guidelines for the appropriate care and use of laboratory animals (IAEC No: KMCRET/Ph.D/16/2016-2017).

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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