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PROTECTIVE EFFECT OF *CAESALPINIA BONDUCELLA* (LINN.) SEED KERNEL EXTRACT IN STREPTOZOTOCIN-INDUCED HYPERGLYCAEMIA AND OXIDATIVE DAMAGE IN WISTAR RATS

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Keywords:

Hyperglycemia; Oxidative stress; Streptozotocin; Ethanolic extract; *Caesalpinia bonducella*

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ABSTRACT: Evidence implicates hyperglycemia induced oxidative stress as a key factor in the pathogenesis of both microvascular and macrovascular complications in the body. Our main objective behind this study was to explore the beneficial effect of *Caesalpinia bonducella* seed kernel extract on Streptozotocin-induced hyperglycaemia and oxidative damage in rats. Rats were made hyperglycemic by single intravenous injection of Streptozotocin 40mg/kg of body weight. Hyperglycemic rats treated with extract in three different doses of 200, 400 and 600 mg/kg of body weight orally for 21 days shows a significant decrease in blood glucose, HbA1c, free amino groups whereas an increase in serum insulin level, liver glycogen contents with a significant improvement in glucose tolerance. Furthermore, above extract supplementation significantly decreases the pancreatic thiobarbituric acid-reactive substances levels along with an increase in superoxide dismutase, catalase and glutathione contents in pancreatic tissues in treatment groups when compared with control hyperglycemic rats. Treatment with above extract has lessened the severities of degenerative changes in pancreas and also in the liver tissues as compared to that of untreated control rat with hyperglycemia. Our results suggest that the ethanolic extract of *Caesalpinia bonducella* seed kernel improves the antioxidant defense under hyperglycemic condition and also exhibit strong anti-hyperglycaemic effect in dose dependent manner.

INTRODUCTION: Diabetes mellitus (DM), a metabolic disorder characterized by chronically elevated blood sugar level either due to impaired insulin secretion, or altered glucose utilization in the vital organs or both, has reached epidemic proportions globally. [1, 2]

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Around 376 million diabetic patients are anticipated worldwide by 2030 and about two billion people will be at risk due to altered lifestyle and lack of definite cure. [3] Thus, it is needed to speed up the ongoing research targeting diabetic patho-mechanism and therapeutics.

Multiple experimental works exploring the reasons behind development and progression of the DM, have shown critical involvement of oxidative stress in the disease pathology. [4, 5] Persistent hyperglycemia itself as well as via formation of

Advanced Glycation End products (AGEs) and its interaction with RAGE receptor, is reported to induce generation of Reactive oxygen species (ROS) such as free radicals; superoxides etc. and/or bring about changes in antioxidant capacity. [6, 7] The impaired cellular redox system is evidenced preclinically as well as clinically, to in turn activate various pathways including polyol pathway flux, AGE formation, and hexosamine pathway flux, and activation of protein kinase-C etc., mediating the development and aggravation of diabetic complications. [8] Further, various endogenous and exogenous antioxidant agents, in addition to many other phytochemicals present in food possessing antioxidant activity are found to be protective against the deleterious effects of free radical species during diabetes. [9-11]

Presently due to unavailability of definite cure for DM, lifelong maintenance therapy is required which has its own pros and cons. Thus it has raised the need to search for safer and equally efficacious alternatives, which may be one of the reasons for diversion of the interest of scientists worldwide towards herbal system of medicine. Large body of work have evidenced traditionally used medicinal plants especially those with antioxidant properties like *Cassia auriculata*, *Nigella sativa*, *Allium sativum*, to prevent several chronic diseases like cancer, asthma etc., have immense therapeutic potential for DM as well. [12-15] Additionally, flavonoids, existing as secondary plants metabolites apart from antioxidant, antihyperglycemic, antihyperlipidemic, and anti-inflammatory properties were also found to possess anti-allergic, anti-viral, anti-bacterial, and anti-tumor effects which raises their clinical usefulness as a treatment for diabetes and related complications.[16, 17]

In search of herbal bioactive devoid of any undesired side effect and toxicity, the number of studies exploring therapeutic potential of traditionally used medicinal plants is increasing although. But, the data pertaining to systematic screening of majority of these drugs for their therapeutic benefits as well as related to mechanism accounting for their beneficial role in diabetes and in glycation induced complications have yet not been extensively reported.

One of such traditionally used medicinal plant is *Caesalpinia bonducella* Linn. (Fever nut) of family Caesalpiniaceae commonly known as Nata-Karanja, is a prickly shrub widely distributed throughout the hotter parts of India, Sri Lanka and other tropical regions of the world.[18, 19] Its seed is grey, hard, globular shaped with a smooth shining surface and consists of a thick, brittle shell with a yellowish white bitter fatty kernel. The plant is well known for its medicinal and therapeutic values in Indian system of medicine. The crude extracts of *Caesalpinia bonducella* seed already been used in folk medicines and powdered seed kernel is also used in the treatment of diabetes by locals in Assam, India.[20] It has been reported to have anti-inflammatory, antipyretic, analgesic, antioxidant, antibacterial, antifilarial, antiasthamatic, antitumor, anxiolytic[18], immunomodulatory and hepatoprotective properties.[21-25] Some other study reports also confirmed the hypolipidemic action of the seed extract and additional benefits in the diabetic condition. Apart from that there are some reports showing hypoglycemic and antidiabetic activity of the extracts of its seeds kernel. [26, 27]

Phytochemical analysis of seeds has revealed the presence of alkaloids, flavonoids, glycosides, saponins, tannins and triterpenoids.[28] The seed contain fatty acids like palmitic acid, stearic acid, oleic acid, a noncrystalline bitter glycoside bonducin. It also contains phytosterins, caesalpins, bonducellin, citrullin, in addition to some important constituents including lupeol,αand β-amyrin.[23] The kernel contains fatty oil, starch, sucrose two phytosterols, one of them is identified as sitosterol like ß-sitosterol, a secondary metabolite, which is a bioactive compound.[18]

Keeping the above facts in consideration, the main purpose of this study was to make systematic investigation on the therapeutic potential of *Caesalpinia boducella* seed kernel extract in hyperglycemia and related complications. Accordingly, our study was designed to explore the plausible mechanism of anti-diabetic action collectively with serum insulin assay to determine the insulin releasing properties of the above extract.

2. MATERIAL AND METHODS:

2.1 Chemicals and reagents:

Glutathione oxidized (GSSG) and reduced (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 5,5 -dithiobis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), and 2,4-dinitrophenyhydrazine (DNPH), Citric acid, Sodium citrate, Tris HCl Buffer AR (Trizma), and Streptozotocin were procured from Sigma–Aldrich Co. (St. Louis, MO, USA). Gliclazide tablets were purchased from the Appolo Pharmacy, (Manufactured by Panacea Biotech Private Ltd., Baddi (H.P.) India. All the other chemicals and reagents used for the experiment were of highest purity (analytical grade) and commercially available.

2.2 Plant material:

The seeds of *C. bonducella* Fleming were collected in the month of July and August from the Herbal Garden at Nizamia Unani Medical College Gaya, Bihar (India). The plant material was authenticated by Dr. Vidhu Aeri, Professor at Department of Pharmacognosy and Phytochemistry, faculty of Pharmacy, Hamdard University, New Delhi. A voucher of specimen number DPP/H/PG-01/2011 has been deposited at the Faculty of Pharmacy, Hamdard University, New Delhi (India).

2.3 Phytochemical studies:

Phytochemical investigation of powdered seed kernel of *C. bonducella* was conducted for the presence of flavonoids (Shinoda's test), glycosides (Molisch's test), reducing sugars (Fehling's test), saponins (foam test) and triterpinoids by Liebermann-Burchard test. [29]

2.4 Preparation of the extract:

The seed kernels of *C.bonducella* Fleming were manually separated from the outer seed shell; air dried and was grounded to a coarse powder. The coarse powder was subjected to phytochemical screening to check the presence of flavonoids. Powdered drug (800g) was defatted with petroleum ether (60–80 °C) by using Soxhlet apparatus, for 16 hours. After defatting, the powdered material was air dried and extracted with 99% ethanol in a Soxhlet apparatus. The extract was dried in a vacuum rota-vapour, giving a brown color residue (yields 8.5 % w/w).

2.5 HPTLC profiling of ethanolic extract of *Caesalpinia bonducella* seed kernel:

Through thin layer chromatograhpy (TLC) different mobile phases were tried for this purpose in different ratios. Various mobile phases that were tried are methnol-chloroform, 7:3 (%v/v), Toluene-ethylacetate 8.5:1.5 (%v/v), n-hexane–2-propanol, 5:5 (%, v/v), n-hexane–2-propanol–acetone, 4:4:2 (%, v/v), Tolune-Ethyl acetate-Formic acid 7:2:1 (%v/v/v), n-butanol-ethylacetate-formic acid-water 4:4:1.2:1 (%v/v/v/v), acetone-toluene-formic acid 4:5:1 (%v/v/v), ethylacteate-methnol-formic acid 5:4:1 (%v/v/v), chloroform-methnol-formic acid 6:4:0.2 (%v/v/v), toluene-ethylacetate-formic acid 5:4:1 (%v/v/v), and toluene-ethylacetate-formic acid 7:2:1 (%v/v/v).

Finally the mobile phase comprising toluene-ethylacetate-formic acid 7:2:1 (%v/v/v) was selected and optimized. HPTLC finger printing profile of ethanolic extract was done at 270nm by using optimised solvent system comprising of Toluene: Ethyl acetate: Formic Acid 7:2:1 (%v/v/v). Aluminum backed precoated plates Silica Gel 60 F 254 was used as stationary phase. A simple HPTLC finger printing is reported that can be used for the quality control of marketed preparations containing *C. bonducella* seed kernel extract and its preparation.

TABLE 1: HPTLC FINGER PRINTING OF SEED EXTRACT OF CAESALPINIA BONDUCELLA AT 370NM

Test extract	Optimised Solvent system	Total Peaks	R_f Values	%Area
Ethanol	Toluene:Ethylacetate:Formic acid 7:3:1 (%v/v/v)	15	0.02, 0.04, 0.06	47.43
			0.41, 0.43, 0.45	1.78
			0.49, 0.51, 0.53	1.24
			0.53, 0.56, 0.58	0.62
			0.55, 0.57, 0.59	2.37
			0.67, 0.69, 0.69	1.63
			0.69, 0.71, 0.73	1.20
			0.75, 0.76, 0.78	1.71
			0.77, 0.78, 0.80	2.28

	0.78, 0.79, 0.81	3.14
	0.81, 0.84, 0.86,	14.17
	0.88, 0.88, 0.90	0.63
	0.92, 0.95, 0.96	0.29
	0.95, 0.95, 0.96	2.62
	0.96, 0.96, 0.97	0.48

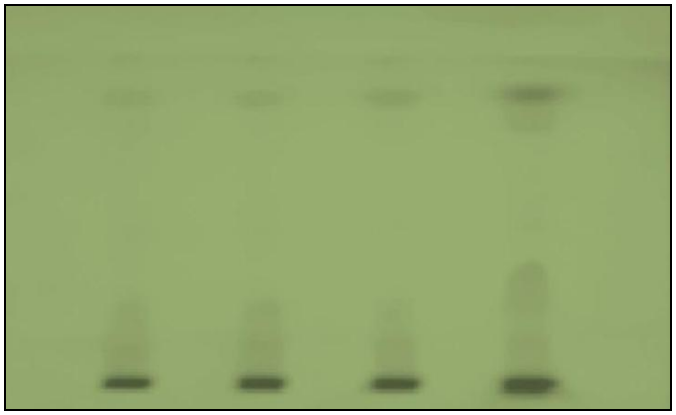


FIG.1: HPTLC FINGER PRINTING PROFILE OF ETHANOLIC EXTRACT OF *CAESALPINIA BONDUCELLA* SEED KERNEL AT λ=270NM

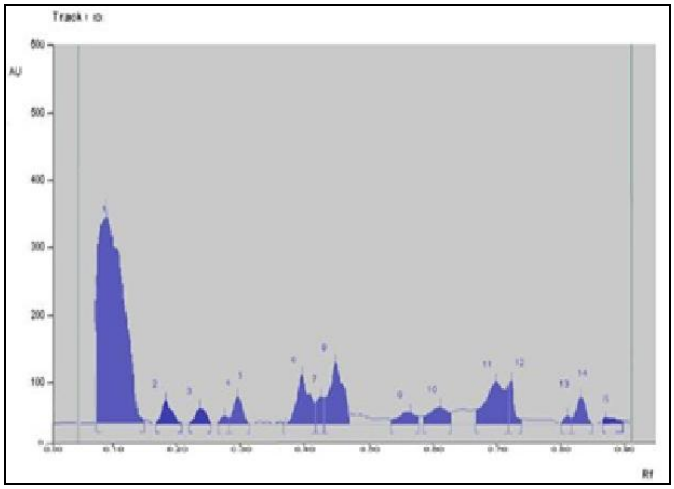


FIG. 2: A HPTLC CHROMATOGRAM OF ETHANOLIC EXTRACT OF *CAESALPINIA BONDUCELLA* SEED KERNEL AT λ=270NM

2.6 Preparation of dosage form:

Ethanolic extract of Seed Kernel of *C.bonducella* in accurately weighed quantities were suspended in 1% Sodium carboxy methyl cellulose (NaCMC) to prepare suitable dosage form. The oral dose of the ethanolic extract of *C. bonducella* seed kernel was determined from previous studies. [18]

2.7 Gliclazide Suspension:

The weighed amount of Gliclazide (25mg/kg, body weight) was freshly made into suspension using 0.05% Sodium carboxy methyl cellulose (NaCMC) and distilled water as a vehicle.

2.8 Animals:

56 Wistar albino rats of either sex weighing between 150-300 g were obtained from Central Animal House Facility of Jamia Hamdard, New Delhi. Animals were housed in polypropylene cages under Standard laboratory conditions (i.e. 12 hours light/12 hours dark cycle; 25±3^{0}C, humidity (35%-60%) were maintained throughout the experimental period, on Standard pellet diet (Aashirwad Ltd. Chandigarh, India) and water *ad libitum*. The present study conforms to the guidelines and principles, in accordance with CPCSEA (173/CPCSEA, 28th Jan 2000) given by Institutional Animal Ethics Committee (IAEC), Jamia Hamdard, Hamdard Nagar, New Delhi-110062.

2.9 Experimental design:

Animals were divided into seven groups comprising of 8 animals in each group as follows:

Group-I: Normal control (untreated rats) given intraperitonial injection of Normal saline (1.0ml/kg), for 21 days. This group served as Normal Control.

Group-II: Hyperglycemic rats (Streptozotocin 40 mg/kg, body weight), served as hyperglycemic Control.

Group-III: *Per Se* group, rats in this group were given only the ethanolic extract of *C.boducella* seed kernel in the the dose of 400 mg/kg, body weight for 21 days. This group was used to know the *per se* effect of the drug.

Group-IV: Hyperglycemic rats given ethanolic extract of *C. boducella* seed kernel in the dose of 200 mg/kg, body weight orally for 21 days.

Group-V: Hyperglycemic rats given ethanolic extract of *C. boducella* seed kernel in the dose of 400 mg/kg, body weight, orally for 21 days.

Group-VI: Hyperglycemic rats given ethanolic extract of *C. boducella* seed kernel in the dose of 600 mg/kg, body weight orally for 21 days.

Group-VII: Hyperglycemic rats given the standard drug gliclazide in the dose of 25 mg/kg, body weight for 21 days.

2.10 Induction of hyperglycemia:
Overnight fasted animals were made hyperglycemic by a single intravenous injection of Streptozotocin (STZ, Sigma, 40mg/kg, i.v. dissolved in 0.05 M citrate buffer, pH-4.5 via the tail vein. Hyperglycaemia was confirmed by the elevated blood glucose levels in plasma, determined at 72 hours after streptozotocin injection. Rats with fasting plasma glucose level 250 mg/dl or higher were considered to be diabetic and were used in the study.

2.11 Tissue Preparation:
At the end of experiment, rats were anesthetized by ether inhalation and rats were then sacrificed. Rat pancreatic tissues and liver were removed immediately and perfused with ice-cold saline. To prevent auto-oxidation or ex vivo oxidation of the tissue, homogenization was carried out at 4 ◦C with 10 times (w/v) in 0.1M phosphate-buffer (pH 7.4). Then the tissue homogenate was centrifuged at 3000 rpm for 5min at 4 ◦C to separate the nuclear debris and was used for estimation of thiobarbituric reactive substances (TBARS). The supernatant was further centrifuged at 10,000×g for 20 min at 4 ◦C to get the post-mitochondrial supernatant (PMS), which was used for various biochemical assays as per reported procedures.

2.12 Analytical procedures:
2.12.1 Body weight measurement:
The body weights of the rats before (on '0' day) and after (on 21st day) drug administration were compared using Student's't' test (Paired).

2.12.2 Oral glucose tolerance test (OGTT):
Oral glucose tolerance test was performed to assess the glucose tolerance according to method of Pari and Sarvanan, 2002. [30] For this purpose glucose solution (2 g/kg) was administered to overnight fasted rats from all the groups. Blood samples were taken at 0, 30, 60, 90 and 120 min. intervals from orbital sinus for glucose estimation.

2.12.3 Determination of blood glucose:
Blood glucose level was estimated by glucose oxidase method (Braham and Trinder, 1972) using a commercial diagnostic kit from Span Diagnostic Limited, Surat, India.[31]

2.12.4 Determination of HbA1c level:
Glycosylated haemoglobin (HbA1c) level was estimated by method of Trivelli *et al.,* 1971 using a commercial diagnostic kit from APBIO, Asritha Diatech, India. [32]

2.12.5 Determination of insulin level:
Serum insulin level was estimated quantitatively by ELISA method (Kratzsch *et al.,* 1990). For this purpose insulin ELISA kit was used. [33]

2.12.6 Estimation of liver glycogen content:
Liver glycogen content was determined by the method of Hassid and Abraham, 1957.[34] Immediately after excision from the animal, approximately 0.5g of liver tissue was digested in 10ml of 30% potassium hydroxide for 20 minutes on boiling water bath. 1ml of this solution was taken and to this 1ml of saturated solution of sodium sulphate (100g sodium sulphate in 150 ml water) was added followed by 2ml alcohol. The solution was then cooled in ice for 15 minutes and centrifuged. Supernatant liquid was discarded and precipitate was dissolved in 4.5ml of distilled water. Glycogen was reprecipitated by adding 2ml alcohol. Centrifuged again, supernatant was discarded and precipitate was dissolved in 10ml distilled water.

This solution contained glycogen. 3ml of this solution was taken and to this 2ml water, 0.1ml 80% phenol (1.6g phenol in 2ml distilled water) followed by 5ml of concentrated sulphuric acid was added, a reddish brown colour appeared. Standard glycogen solution was prepared by taking 10 mg of pure standard glycogen that was dissolved in 50ml of distilled water, giving a concentration of 200µg/ml. 3ml of standard glycogen solution was taken and to this 2 ml water was be added and 0.1ml 80% phenol followed by 5ml of concentrated sulphuric acid, reddish brown colour appeared. The

optical density was read within 2 h in a spectrophotometer at 620 nm against a blank that was prepared by subjecting 5 ml of distilled water instead of sample to the same procedure.

2.12.7 Estimation of free amino groups (in serum & pancreatic tissues):

The free amino groups of protein were estimated both in serum and pancreatic tissues by the method of Habeeb 1966, with slight modification.[35] Suitable aliquots of serum and tissue samples separately were dissolved in 1.0 ml of 0.1 M sodium tetraborate buffer, pH 9.3 and 25µl of 0.3 M TNBS was added. The tubes were agitated instantly to ensure complete mixing and it was allowed to stand for 30 minutes at room temperature. Absorbance was read at 420nm.

2.13 Biochemical estimation in pancreatic tissues:

2.13.1 Assay for protein estimation:

Protein content in all samples was estimated by the method of (Lowry *et al.,* 1951) using bovine serum albumin (BSA) as a standard. [36]

2.13.2 Assay of TBARS content:

TBARS content was measured by the method of Ohkawa *et al.,*1979 with some modification.[37] Briefly, 1 ml of suspension medium was taken from the supernatants of the 10% tissue homogenate and centrifuged at 10,000 rpm 0.5 ml of 30 % TCA followed by 0.5ml of 0.8 % TBA was added to it. The tubes were covered with Aluminium foil and kept in shaking water bath for 30 minutes at 80° C. After 30 minutes, tubes were taken out and kept in ice-cold water for 10 minutes. Finally it was centrifuged at 3000 rpm for 15 minutes. The absorbance of supernatants was read at 540 nm at room temperature against appropriate blank. Blank consists of 1.0 ml distilled water, 0.5 ml TBA, and 0.5 ml TCA.

2.13.3 Assay for reduced glutathione:

GSH content was determined by the method of Sedlak and Lindsay, (1968).[38] Briefly a known weight of tissue ranging from (300-600 mg) was homogenized in 5-8 ml of 0.02 M EDTA and then 4.0 ml of cold distilled water was added to it. After mixing it well, 1ml of 50% trichloroacetic acid (TCA) was added and shaken intermittently for 10 minutes using a vortex mixer. After 10 minutes the contents was transferred to centrifuge tubes (rinsed in EDTA) and centrifuged at 6000 rpm for 15minutes. Following centrifugation, 2ml of the supernatant was mixed with 4.0ml of 0.4 M Tris buffer (pH 8.9). The whole solution was mixed well & 0.1ml of 0.01M DTNB (5, 5-Dithiobis-2-nitrobenzoic acid) was added to it. Absorbance was read within 5 min of the addition of DTNB at 412nm against a reagent blank. The GSH concentration was calculated as nmol DTNB conjugate formed/gm tissue.

2.13.4 Assay for SOD activity:

SOD activity was measured by the method of Marklund and Marklund, (1974).[39] The supernatant was assayed for SOD activity by following the inhibition of pyrogallol autoxidation. 100µl of cytosolic supernatant was added to Tris HCl buffer (pH 8.5). The final volume of 3 ml will be adjusted with the same buffer. At last 25µl of pyrogallol was added and changes in absorbance at 420 nm were recorded at 1 minute interval for 3 minutes. The increase in absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD.

2.13.5 Assay for catalase activity:

Catalase activity was assayed by the method of Claiborne (1985). [40] Briefly the reaction mixture consists of 1.95 ml phosphate buffer (0.1M, pH 7.4), 1.0 ml hydrogen peroxide (0.019M) and 0.05 ml PMS in a final volume of 3 ml. Changes in absorbance was recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed/min/mg protein.

2.14 Histopathological study of pancreas and liver tissues:

Histopathology was done by the method of Belur *et al.,* (1990).[41] For histological examinations, pancreas and liver were isolated and preserved in 10% neutral buffered formalin. Histopathological observation of tissues was carried out at Pathology laboratory, HIMSR, Jamia Hamdard (New Delhi, India). After fixation, the tissues were embedded in paraffin and clear in xylene and dehydrated in descending series of ethanol. At least four cross-sections were taken from each tissue of 5µm thickness and stained with hematoxylin and eosin

(H&E). Following two changes xylene washes of 2 min each, tissue sections were mounted with DPX mount. All the slides were observed for histopathological changes and photomicrographs were taken using an Olympus BX-51 microscope (Olympus, Japan).

2.15 Statistical analysis:

Data are presented as mean ± SEM (n=7). One-way ANOVA with Dunett's Multiple Comparison post-test was performed to assess differences between means using Graphpad Prism version 4.00 for Windows (Graphpad Software, San Diego, Ca, USA). Values P<0.05 were considered statistically significant.

3. RESULTS:

3.1 Effect on changes in body weight:

TABLE 2: EFFECT OF *C. BONDUCELLA* TREATMENT ON CHANGES IN THE BODY WEIGHT IN DIFFERENT GROUPS OF ANIMALS.

Treatment	Day 0	Day 21
Group I	254.29 ± 10.54	285.00 ± 9.57
Group II	252.14 ± 6.53	199.28 ± 6.4^{**aa}
Group IV	238.57 ± 5.08	257.14 ± 17
Group V	272.14 ± 18.12	297.86 ± 18.18$^{\#\#}$
Group VI	261.42 ± 8.77	287.86 ± 8$^{\#\#a}$
Group VII	234.28 ± 9.6	275.71 ± 10.14$^{\#\#a}$
Group III	204.28 ± 5.5**	233.57 ± 3.73^{aa}

* P < 0.05, ** P < 0.01: significant when compared to Group I; $^{\#}$ P < 0.05, $^{\#\#}$ P < 0.01: significant compared to Group II; a P < 0.05, aa P < 0.01: significant when compared to the Day 0.

Table 2 shows the effect of *C. bonducella* treatment on changes in the body weight. The final body weight showed significant (p<0.05) increase from the initial body weight in all the groups except in Diabetic Control Group, in which there was statistically significant (p<0.05) decrease in body weight compared to the initial body weight.

Oral administration of ethanolic extract of *C. bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, registered a significant (p<0.05) improvement in body weight.

3.2 Effect of Ethanolic extract of *C. bonducella* on blood glucose levels:

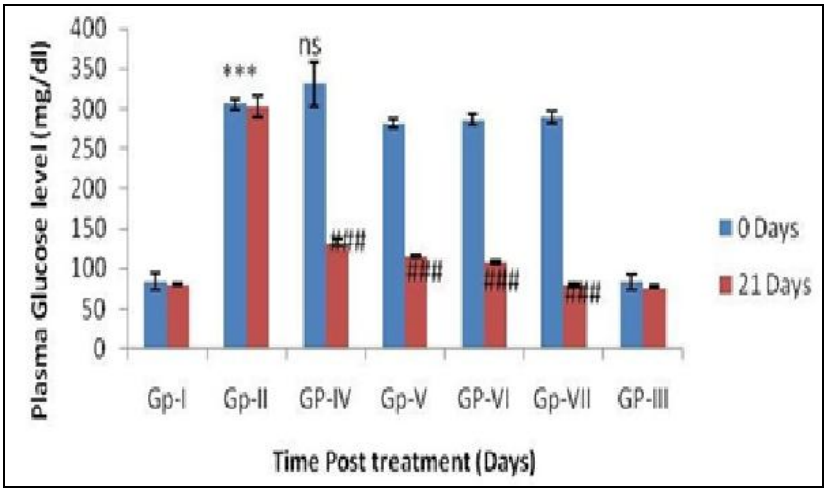


FIG. 3: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT AND GLICLAZIDE POST-TREATMENT ON PLASMA GLUCOSE LEVELS IN DIABETIC RATS.

Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001 shows significant difference in the group IV, V, VI & VII respectively when compared with group II. There was no significant difference observed between group I & III.

Fig. 3 shows the effect of *C. bonducella* on the blood glucose level. Significant increase in blood glucose level was observed in Diabetic control rats when compared to Normal control rats treated with

normal saline. Oral administration of ethanolic extract of *C. bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, reduced the blood glucose level significantly ($p<0.001$) in dose dependent manner. Only *per se* treatment did not register any significant change in the blood glucose level when compared to normal control rats.

3.3 Effect of Ethanolic extract of *C. bonducella* on OGTT:

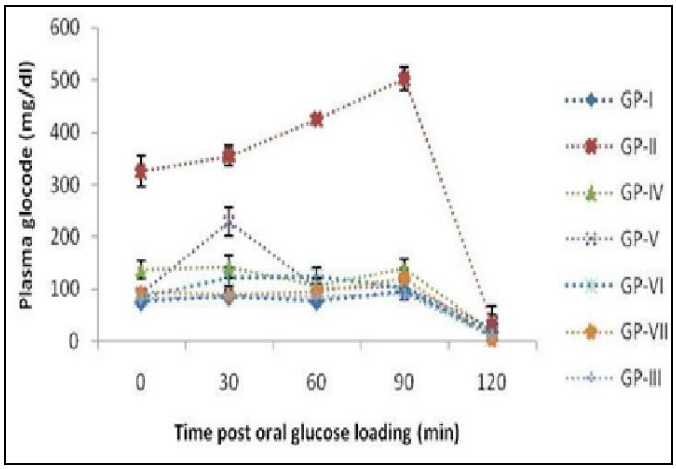


FIG.4: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT AND GLICLAZIDE POST-TREATMENT ON ORAL GLUCOSE TOLERANCE TEST IN ALL THE GROUPS.

Values are expressed as mean ± SEM. (n = 7). $^{***}p<0.001$ shows significant difference in group II when compared with group I. $^{###}p<0.001$, $^{###}p<0.001$, $^{###}p<0.001$, $^{###}p<0.001$ shows significant difference in the group IV, V, VI & VII respectively when compared with group II. There was no significant difference observed between group I and III.

Fig. 4 shows the blood glucose levels of all the groups after oral administration of glucose (2 g/kg). In diabetic control rats, the peak increase in blood glucose level was observed after 30, 60 and 90 minutes. The blood glucose level remained high over next 30 minutes. Ethanolic extract of *C. bonducella* after oral administration in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, showed significant ($p<0.001$) decrease in blood glucose level at 60 and 120 minutes when compared to diabetic control rats. There was no significant change in the blood glucose level observed between per se group and normal control group at 60 and 90 minutes during OGTT.

3.4 Effect of Ethanolic extract of *C.bonducella* on HbA1c level:

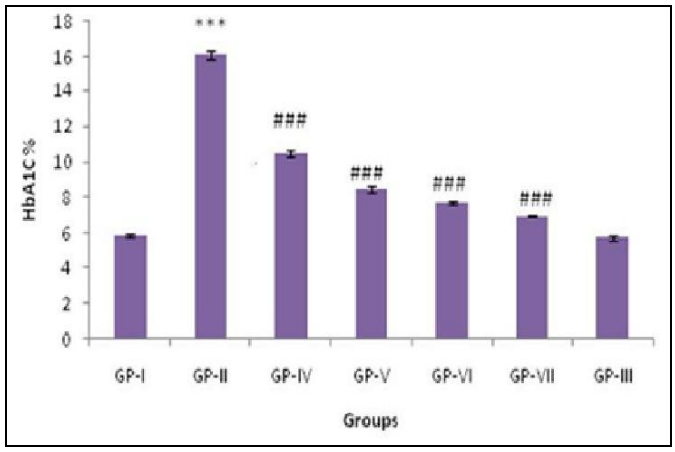
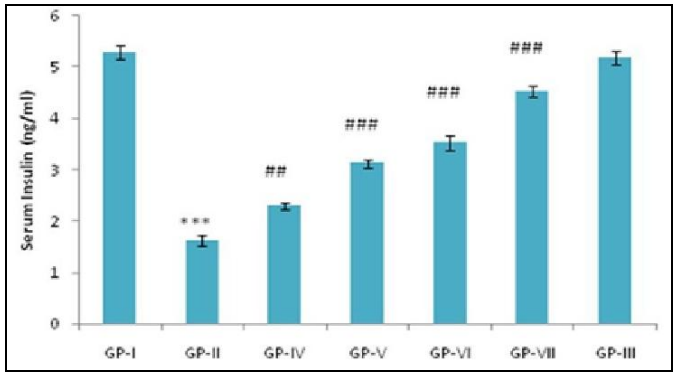


FIG. 5: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT AND GLICLAZIDE POST-TREATMENT ON HBA1C LEVEL IN STREPTOZOTOCIN INDUCED DIABETIC RATS.

Values are expressed as mean ± SEM. (n = 7). $^{***}p<0.001$ shows significant difference in group II when compared with group I. $^{###}p<0.001$, $^{###}p<0.001$, $^{###}p<0.001$, $^{###}p<0.001$ shows significant difference in the group IV, V, VI & VII respectively when compared with group II. There was no significant difference observed between group I and III.

Fig.5 shows the effect of ethanolic extract of *C. bonducella* on HbA1c level. Significant ($p<0.001$) increase in HbA1c level was observed in diabetic control rats when compared to normal control rats. Oral administration of Ethanolic extract of *C. bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, decreased the HbA1c level significantly ($p<0.001$) in a dose dependent manner. There was no significant change in HbA1c level of only per se group rats when compared to normal control rats.

3.5 Effect of Ethanolic extract of *C.bonducella* on serum insulin level:



EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT AND GLICLAZIDE POST-TREATMENT ON INSULIN LEVEL IN STREPTOZOTOCIN INDUCED DIABETIC RATS.

Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. $^{\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001 shows significant difference in the group IV, V, VI & VII respectively when compared with group II. There was no significant difference observed between group I and III.

Fig.6 shows the effect of ethanolic extract of *C. bonducella* on serum insulin level was reduced in diabetic control rats when compared to normal control rats. Oral administration of Ethanolic extract of *C.bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, significantly (p<0.001) increases the levels of insulin when compared to diabetic control rats. Only per se treatment did not induce any change in the level of insulin when compared to normal control rats.

3.6 Effect of Ethanolic extract of *C.bonducella* on liver glycogen content:

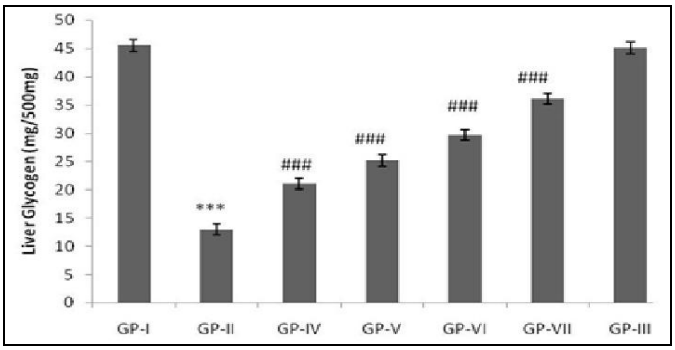


FIG.7: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT AND GLICLAZIDE POST-TREATMENT ON LIVER GLYCOGEN LEVEL IN STREPTOZOTOCIN INDUCED DIABETES.

Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001 shows significant difference in the group III, IV, V & VI respectively when compared with group II. There was no significant difference observed between group I and III.

Fig.7 shows the effect of *Caesalpinia bonducella* extract post-treatment on liver glycogen level in streptazotacin induced diabetes was reduced in diabetic control rats when compared to normal control rats (**Fig.5**). Oral administration of Ethanolic extract of *C.bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, significantly (p<0.001) increased the glycogen content in liver insulin when compared to diabetic control rats. There was no significant difference observed between per se and normal control group.

3.7 Effect of Ethanolic extract of *C. bonducella* on free amino groups (tissue and serum):

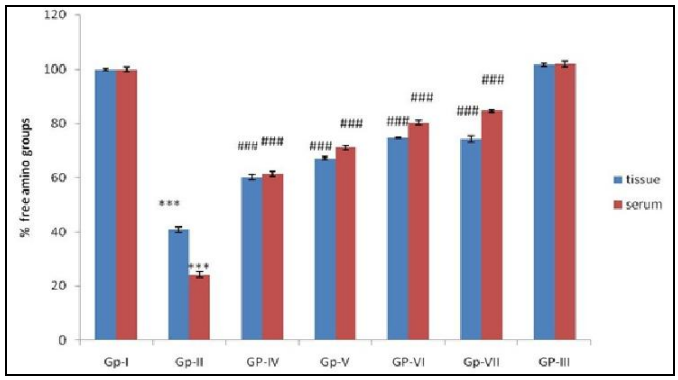


FIG.8: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT AND GLICLAZIDE, POST-TREATMENT ON FREE AMINO GROUPS (TISSUE AND SERUM) IN STREPTOZOTOCIN INDUCED DIABETES. Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001, $^{\#\#\#}$p<0.001 shows significant difference in the group III, IV, V & VI respectively when compared with group II, both in tissue and serum . There was no significant difference observed between group I and III.

Fig. 8 shows the effect of ethanolic extract of *C.bonducella* on free amino groups (tissue and serum). A significant (p<0.001) increase in free amino groups both in tissue and serum was observed in diabetic control rats when compared to normal control rats. Oral administration of ethanolic extract of *C.bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, decreased the % free amino groups (tissue and serum) significantly (p<0.001) in a dose dependent manner. There was no significant change in free amino groups (tissue and serum) in per se group when compared to normal control group.

3.8 Ethanolic extract of *C.bonducella* modulated TBARS contents in hyperglycemic rats:

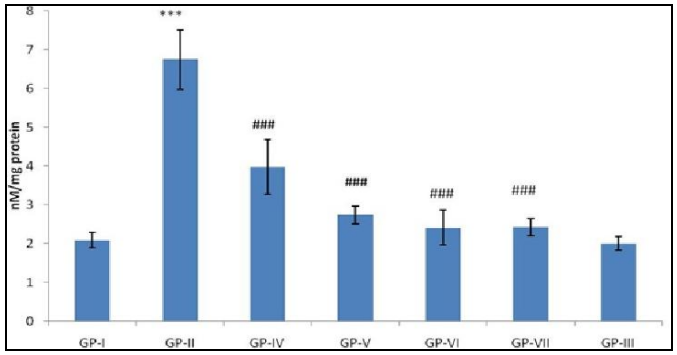


FIG.9: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT POST-TREATMENT ON TBARS LEVELS IN STZ-INDUCED HYPERGLYCEMIC RATS.

Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. ###p<0.001, ###p<0.001, ###p<0.001, ###p<0.001 shows significant difference in the group IV, V, VI & VII respectively when compared with group II. There was no significant difference observed between group I and III. ###p<0.001, ###p<0.001 shows significant difference in the group III, IV, V & VI respectively when compared with group II.

Fig.9 shows the effect of ethanolic extract of *C.bonducella* on TBARS content. A significant (p<0.001) increase in TBARS level was observed in hyperglycemic control rats when compared to normal control rats. Oral administration of ethanolic extract of *C.bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, decreased the TBARS level significantly (p<0.001) in a dose dependent manner. There was no significant change observed in the TBARS level between *per se* and normal control group.

3.9 Ethanolic extract of *C. bonducella* on GSH contents in pancreatic tissues:

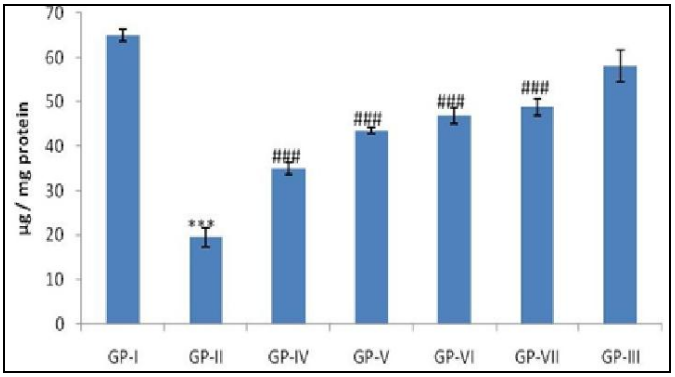


FIG.10: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT POST-TREATMENT ON GSH CONTENTS IN STZ-INDUCED HYPERGLYCEMIC RATS. Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. ###p<0.001, ###p<0.001, ###p<0.001, ###p<0.001 shows significant difference in the group IV, V, VI & VII respectively when compared with group II. There was no significant difference observed between group I and III.

Fig.10 shows a significant (p<0.001) decrease in GSH level was observed in hyperglycemic control rats when compared to normal control rats. After oral administration of ethanolic extract of *C.bonducella* in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, increased the GSH contents significantly (p<0.001) in a dose dependent manner. There was no significant difference in the GSH contents between *per se* and normal control group.

3.10 Effect of *C. bonducella* ethanolic extract on the antioxidant enzymes:

The effect of *C.bonducella* ethanolic extract on STZ-induced alteration in the activities of different antioxidant enzymes (superoxide dismutase and catalase) and the results were shown in **Fig.11** and **Fig.12**.

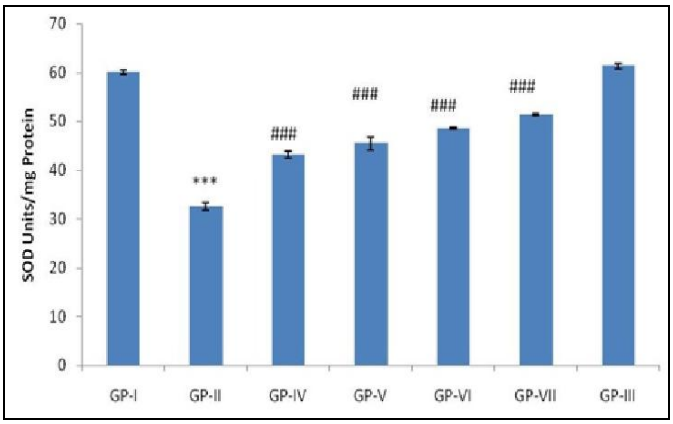


FIG.11: EFFECT OF *CAESALPINIA BONDUCELLA* SEED EXTRACT POST-TREATMENT ON SOD ACTIVITY IN STZ-INDUCED HYPERGLYCEMIC RATS. Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. ###p<0.001, ###p<0.001, ###p<0.001, ###p<0.001 shows significant difference in the group III, IV, V & VI respectively when compared with group II. There was no significant difference observed between group I and III.

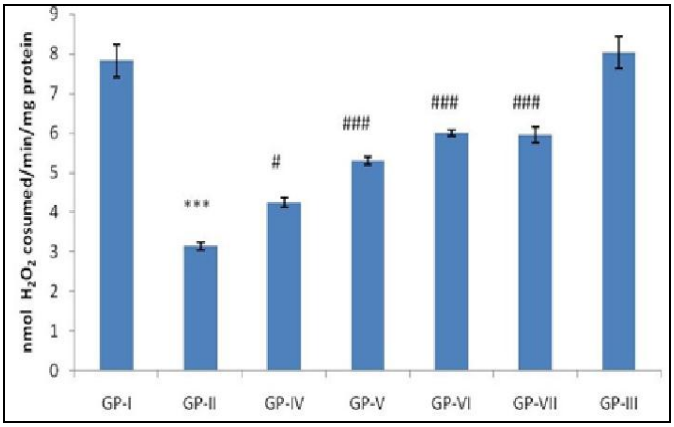


FIG.12: EFFECT OF *CAESALPINIA BONDUCELLA* EXTRACT POST-TREATMENT ON CATALASE ACTIVITY IN STZ-INDUCED HYPERGLYCEMIC RATS. Values are expressed as mean ± SEM. (*n* = 7). ***p<0.001 shows significant difference in group II when compared with group I. #p<0.05, ###p<0.001, ###p<0.001, ###p<0.001 shows significant difference in the group III, IV, V & VI respectively when compared with group II.

We have evaluated that there was a significant ($p<0.001$) difference in the activity of different antioxidant enzymes between in hyperglycemic control rats when compared to normal control rats. Our result shows that oral administration of *C.bonducella* ethanolic extract in the group IV, V, and VI at the dose of 200, 400 and 600 mg/kg respectively, leads to a significant ($p<0.05$, $p<0.01$, $p<0.001$) restoration in the activity of antioxidant enzymes when compared with the in hyperglycemic control rats (GP II). There was no significant difference observed in the activity of antioxidant enzymes between *per se* group and normal control group rats.

3.11 Histopathological findings:

For histopathology study particularly the pancreatic and liver tissues were taken and H&E staining was done to visualize and differentiate between tissue components in normal and diabetic condition as well as treatment gropus. Effects of *C. bonducella* ethanolic extract on STZ-induced alteration in pancreatic tissue and also in liver tissue were examined and the histopathological findings were shown in **Fig. 13** and **14**. Pancreas from normal control group showed normal appearance of pancreatic tissue while the pancreatic tissue from untreated diabetic control group displayed the degenerative changes with a very small sized Islet of Langerhans. *Per se* treatment did not show any remarkable effects when compared with the normal control group. The C. *bonducella* extract supplementation in group IV, V and VI (in a dose of 200, 400 and 600 mg/kg) lessened the severity of degenerative changes when compared with diabetic control rats. Diabetic rats (GP VII) treated with gliclazide (standard) at the dose of 25 mg/kg, showed the pancreatic tissue with normal sized islet of Langerhans.

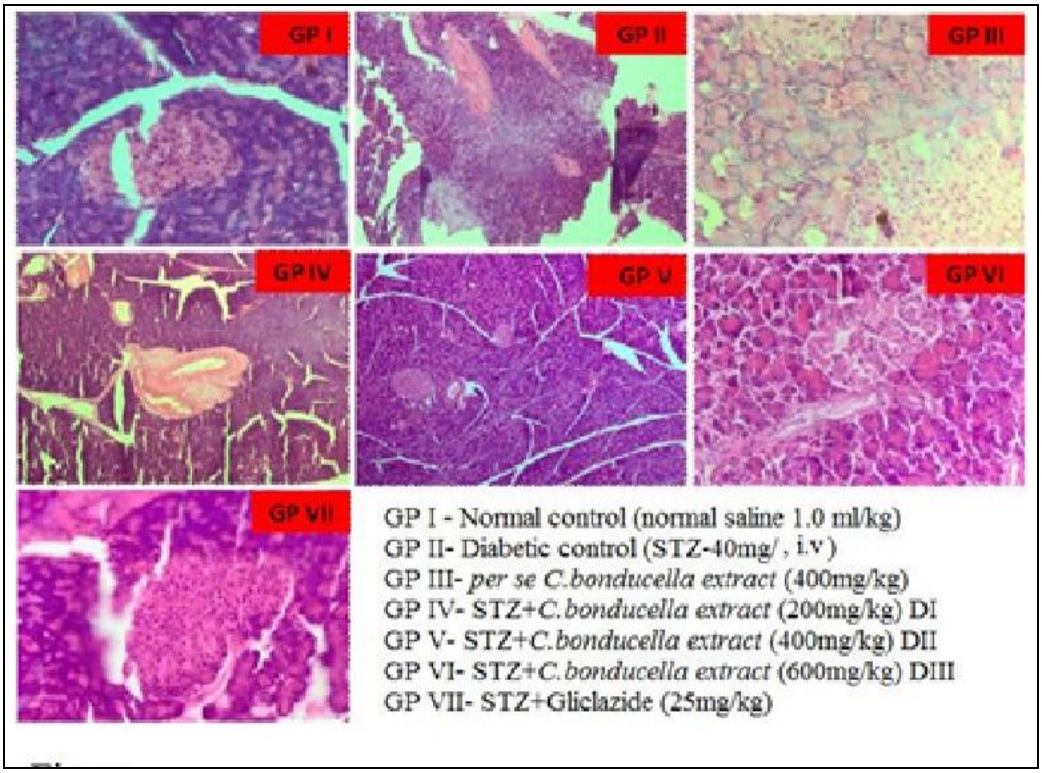


FIG.13: EFFECT OF *CAESALPINIA BONDUCELLA* SEED KERNEL EXTRACT SUPPLEMENTATION ON H & E STAINING IN THE PANCREATIC TISSUE SECTIONS. (GP I) Low power photomicrograph of pancreatic tissue from normal control group showing normal appearance of tissue architecture with a single islet of Langerhans seen in the centre. The Islet size is also within the normal range (H&EX100). (GP II) Low power photomicrograph of pancreatic tissue from untreated diabetic control group displaying degenerative changes in pancreatic tissue with a very small sized Islet of Langerhans. The exocrine tissue is within normal limits (H&E X 100). (GP III) Low power photomicrograph of pancreatic tissue from *per se* group rat showing normal appearance of pancreatic tissue with a single Islet of Langerhans seen in the lower corner (H&E X100). (GP IV) Low power photomicrograph of pancreatic tissue from diabetic rats treated with *C. bonducella* extract=200 mg/kg, showing a small Islet of Langerhans seen in the centre (H&E X100). (GP V) Photomicrograph of pancreatic tissue from diabetic rats treated with *C.bonducella* extract=400 mg/kg, at low power showing a small Islets of Langerhans (H&E X 100). (GP VI) Photomicrograph of pancreatic tissue from diabetic rats treated with *C.bonducella* extract=600 mg/kg, at low power showing pancreatic tissue with a larger islets of Langerhans seen in the centre (H&E X 100). GP VII Diabetic rats treated with gliclazide=25 mg/kg, showing pancreatic tissue with a normal sized islet of Langerhans (H&E X 400).

On the other hand histology of the H&E-stained control liver section (**Fig. 14**), showed normal architecture of hepatocytes. Liver section of STZ-induced hyperglycemic control group showed the degenerative changes comprising of cloudy swelling/hydropic changes of cytoplasm as pyknotic nuclei of liver cell (H&EX100). The C. *bonducella* extract supplementation in group IV, V and VI have lessened the severity of degenerative changes when compared with diabetic control rats. Ethanolic extract supplementation did not show any notable effects in the *per se* group treated with the above extract alone when compared with the normal control group rats.

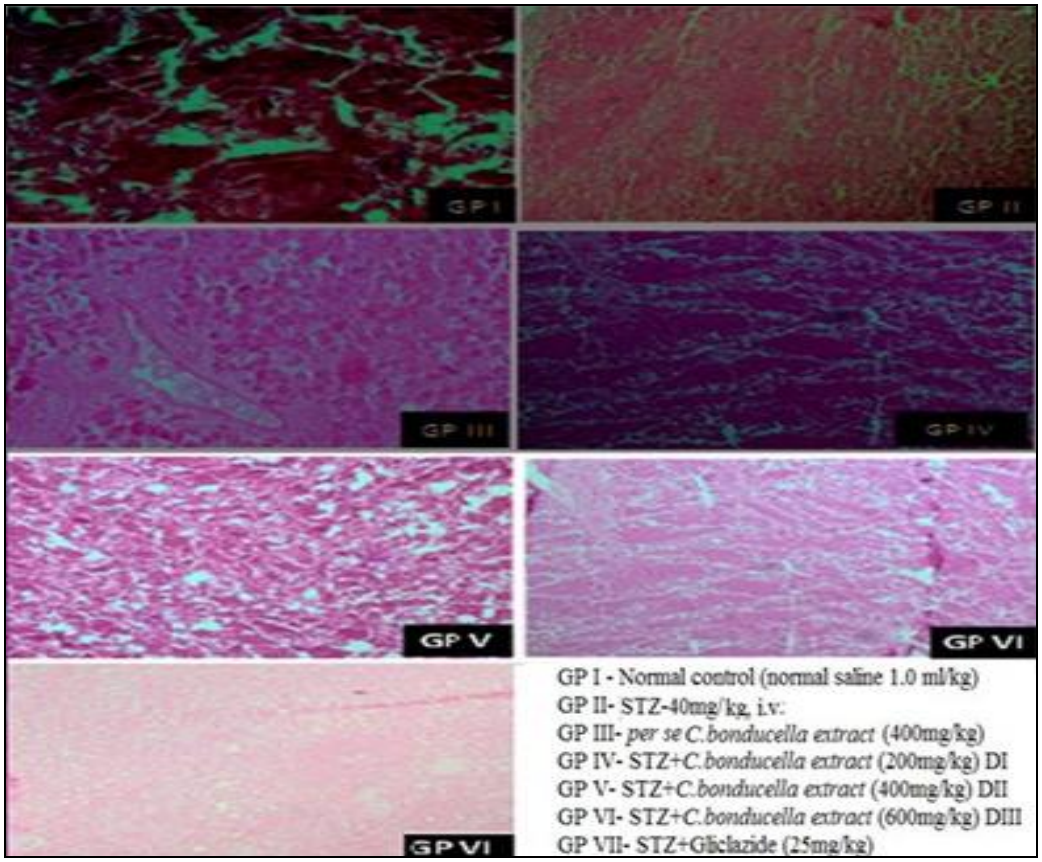


FIG.14: EFFECT OF *CAESALPINIA BONDUCELLA* SEED KERNEL EXTRACT SUPPLEMENTATION ON H & E STAINING IN THE LIVER SECTIONS. Photomicrograph from the normal control group as GP-I normal liver showing hepatic cell with normal cytoplasm and nucleus without any abnormality showing normal liver architecture at lower power-X100. Hyperglycemic control (GP-II) STZ-treated pathogenic group liver section showing the degenerative changes comprising of cloudy swelling/hydropic changes of cytoplasm as pyknotic nuclei of liver cell (H&EX100). GP-III (*per se* group) Hepatic cells without any degenerative changes of nucleus and cytoplasm, thereby revealing well protective effect (H&EX100). GP-IV (STZ+EECBSK=200mg/kg) liver section with minimal degenerative changes of hepatocytes, with swelling without any necrosis thereby revealing moderate reversibility showing well protective effect (H&E X100). GP-V (STZ+EECBSK=400mg/kg) rat liver section showing intact hepatic cells with nucleus and nuclei, cytoplasm without any abnormality, moderate reversibility showing protective effect (H&E X100). GP-VI (STZ+EECBSK=600 mg/kg) liver section showing intact hepatic cells with nucleus and nuclei, cytoplasm without any abnormality, moderate reversibility revealing protective effect (H&E X100). GP-VII (STZ+Gliclazide=25mg/kg) liver section showing normal hepatic cells without any abnormality, revealing well protective effect (H&E X100).

DISCUSSION: Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS) which are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems. [42] The levels Free radicals and other reactive oxygen and nitrogen species that are generated in vivo, are modulated by a network of antioxidant defense systems, assisted by certain repair systems. [5] Tissue injury in human disease is accompanied by an imbalance in the oxidant/antioxidant status, creating oxidative stress. The resulting increased oxidative damage to biomolecules may play an important role in the pathology of several human diseases and is amenable to therapeutic intervention with appropriate antioxidants. [43, 44] There are convincing experimental and clinical evidences that the generation of reactive oxygen species is increased in both types of diabetes and that the onset of

diabetes is closely associated with oxidative stress. [45, 46] Free radicals are formed disproportionately in diabetes by glucose auto-oxidation, polyol pathway and non-enzymatic glycation of proteins. Abnormally high levels of free radicals and simultaneous decline of antioxidant defence systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of diabetes mellitus. The irreversible changes that occur as a result of hyperglycemia are the formation of glycosylated conjugates like HbA1c. Glucose can react with a free amino group, such as the ε-amino group of a protein lysine residue, to form an adduct commonly referred to as a Schiff bases which lead to advanced glycation end products. [47]

These chemically heterogeneous compounds are known to have a wide range of chemical, cellular and tissue effects implicated in the development and progression of diabetes and ageing. [48] Because of the therapeutic limitations of hypoglycaemic therapy in practice, further interventional strategies must be developed for the treatment of diabetes mellitus. In addition to elevated blood glucose levels, increased production of reactive oxygen species (free radicals), which are known to exhibit direct tissue damaging properties, may contribute to a number of diabetic complications and to the development of insulin resistance itself. Therefore, in addition to control of blood sugar, control of oxidative stress offers another avenue for the treatment of the disease.

In the present study, we have examined the oxidative stress pathway markers in the diabetic as well as in normoglycemic rats. These beneficial effects of ethanolic extract of *Caesalpinia bonducella* provide additional support to the findings from previous studies of its free radicals scavenging activity with powerful antioxidant properties. In diabetes, there is an increased glycosylation of a number of proteins, including hemoglobin. Estimation of HbA1c has been found to be particularly useful in monitoring the effectiveness of therapy in diabetes. From the study, it was seen that ethanolic extract of *C. bonducella* significantly ($p < 0.001$) lowered the blood glucose level in diabetic rats. Streptozotocin a β-cytotoxic agent, rapidly and selectively accumulates in pancreatic β-cells and causes β-cell death and apoptosis by generation of reactive oxygen species, superoxide radicals and hydrogen peroxide. [49, 50]. Free radicals are formed disproportionately in diabetes by glucose auto-oxidation, polyol pathway and non-enzymatic glycation of proteins. High levels of free radicals and immediate decline of antioxidant defence systems can lead to the damage of cellular components including various enzymes, increased lipid peroxidation and development of complications of diabetes mellitus. Antioxidant enzymes scavenge or eliminate a variety of Reactive Species together with those generated during biological processes. [51, 52]

Some important endogenous antioxidant enzymes and antioxidant substance are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). In which SOD maintains the cellular levels of $O_2^{\bullet -}$ within the physiological concentrations by converting $O_2^{\bullet -}$ to H_2O_2, a more stable reactive oxygen species whereas CAT metabolizes H_2O_2 to O_2 and H_2O. Catalase exerts two enzymatic activities, depending on the concentrations of its substrate H_2O_2. It elicits a catalytic function at high concentrations of H_2O_2, whereas it produces a peroxidatic effect at lower concentrations of H_2O_2. [51, 53, 54] GPx enzymatically reduces H_2O_2 to O_2 and H_2O using a hydrogen donor, glutathione (GSH) which is oxidized to glutathione disulfide (GSSG). Hence, the primary device of the cell is to use superoxide dismutase, catalase, and glutathione peroxidase to prevent superoxide anion or hydrogen peroxide from participating in reactions that generate more reactive oxidants, such as hydroxyl radical.[55, 56]

The antioxidant activity of the seed kernel of *Caesalpinia bonducella* might be attributed due to the presence in flavonoids, known to be natural antioxidants, which protect the existing liver cells from damage by their free radical scavenging action. Further, previous studies have also reported the protective action of flavonoid, triterpenoids present in the seed kernel of *Caesalpinia bonducella* that might have the ability to protect cells and tissues from oxidative damage by various mechanisms. Few reports also suggest that the

Triterpenoids (lupeol), present in the seed kernel of *Caesalpinia bonducella*, have ability to protect cells and tissues from oxidative stress by increasing the transcriptional activity of NRF_2, which induces the formation of cytoprotective enzymes like catalase, superoxide dismutase, etc. [57] The antidiabetic effect of *Caesalpinia bonducella* extract can be linked to more than one mechanism. Raised blood glucose level is the principle stimulus for insulin secretion. The fact that the ethanolic extract of seed kernel of *Caesalpinia bonducella* has lowered raised blood glucose level, asserts the presence of some constituents which might have directly lowered the blood glucose level independent of insulin secretion. However, the probability of an insulin releasing action of the constituents of *Caesalpinia bonducella* cannot be ruled out.

The antidiabetic activity of the seed kernel of *Caesalpinia bonducella* might be attributed due to the presence in flavonoids, known to be natural antioxidants, which protect the existing β-cells from dying by their free radical scavenging action. Further, previous studies have reported the protective action of flavonoids against oxidative stress induced cellular damage and also the ability of flavonoids to regenerate β-cells. Triterpenoids present in the seed kernel of *Caesalpinia bonducella*, have the ability to protect cells and tissues from oxidative stress by various mechanisms. The administration of *Caesalpinia bonducella* at different doses significantly lowered the TBARS levels and increased SOD, CAT and GSH levels in a dose dependent manner. The results suggested the antioxidant property of *Caesalpinia bonducella.*

The effect of *C.bonducella* extract on the body weight reduction in STZ-diabetic rats is statistically significant ($p<0.05$) in this study. Loss of body weight is one of the symptoms of diabetes. This loss of body weight in diabetes is due to increased lipolysis and increased muscle wasting and loss of tissue proteins caused by insulin deficiency. *C.bonducella* seed extract, possibly due to its insulin releasing action of its ingredients probably prevented this lipolysis and proteolysis by ameliorating the extent of insulin deficiency and thereby caused an increase in body weight. The administration of *Caesalpinia bonducella* at different doses significantly lowered the TBARS levels and increased SOD, CAT and GSH levels in a dose dependent manner that suggest antioxidant property of *Caesalpinia bonducella.*

These beneficial effects of the extract of *Caesalpinia bonducella* provide additional support to the findings from previous studies of its *in-vitro* free radicals scavenging activity with powerful antioxidant properties. Our findings are also supported by histopathology of the liver tissues of the STZ-treated pathogenic group liver section showing the degenerative changes comprising of cloudy swelling / hydropic changes of cytoplasm as pyknotic nuclei of liver cell (H&EX100). Whereas tissue section from the liver after ethanolic extract supplementation shows intact hepatic cells with nucleus and nuclei, cytoplasm without any abnormality, moderate reversibility revealing protective effect. The present work summarizes the current knowledge of the pathogenic role of oxidative stress in the onset and progression of diabetes and its complications and presents the results of studies that were aimed to modulate oxidative stress through the use of antioxidants in diabetes. These findings corroborate with the earlier reports on antihyperglycemic potential of ethanolic extract of the seeds kernel of *Caesalpinia bonducella*. [27]

However, further investigations are required to project light on the molecular mechanism of possible antidiabetic activity of *Caesalpinia bonducella* seed kernel extract and isolation of pure compounds that will help in identifying the antihyperglycemic principles of *Caesalpinia bonducella* seeds. From the results of present studies, it is also clear that ethanolic extract of the seeds kernel of *Caesalpinia bonducella* has antioxidant and hepatoprotective properties. Besides this many other reports also suggest that the methonolic extract from the leaves of this plant have a significant hepatoprotective property that fulfils its traditional claims that the each part of the plant has medicinal values. [58] As per the standardization part a simple HPTLC finger printing of ethanolic extract of *C. bonducella* is also reported by us that can be used for the quality control of marketed preparations containing *C.*

bonducella. However, further work is warranted to isolate and quantify active constituents that are responsible for its antioxidant potential, together with studies on serum pharmacology to be undertaken.

CONCLUSION: In the present study we have observed that ethanolic extract of *Caesalpinia bonducella* seed kernel reflects protective effects in dose dependent manner by lowering the blood glucose, HbA1C levels, increasing the serum insulin level, free amino groups, liver glycogen, and also demonstrated the antioxidant effect by attenuating the altered oxidative stress parameters in rats with Streptozotocin-induced hyperglycemia. The results suggest that the ethanolic extract of *Caesalpinia bonducella* seed kernel has antihyperglycemic and antioxidant effects. On the basis of the present findings it is concluded that beneficial effect of the ethanolic extract of *Caesalpinia bonducella* seed kernel may be utilized for improving overall antioxidant capacity of the body.

CONFLICT OF INTEREST: The authors declare that there is no conflict of interests regarding the publication of this paper.

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