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Ramesh K Lakshmaiah
Maharani's Science College for Women, Mysuru, Karnataka, India

Manjula M Venkatappa
Department of Studies and Research in Food Science and Nutrition, Tumkur University, Tumkur, Karnataka, India

Shivakumar Venkataramaiah
Departments of Studies and Research in Biochemistry, Tumkur University, Tumkur, Karnataka, India

Rajesh Rangappa
Departments of Studies and Research in Biochemistry, Tumkur University, Tumkur, Karnataka, India

Devaraja Sannaningaiah
Departments of Studies and Research in Biochemistry, Tumkur University, Tumkur, Karnataka, India

Corresponding Author:
Ramesh K Lakshmaiah
Maharani's Science College for Women, Mysuru, Karnataka, India

Aqueous extract of *Cicer arietinum* leaves mitigates the oxidative stress induced RBC and vital organ damage via antioxidant and anti-inflammatory properties

Ramesh K Lakshmaiah, Manjula M Venkatappa, Shivakumar Venkataramaiah, Rajesh rangappa and Devaraja Sannaningaiah

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Abstract
The current study reports the antioxidant, anti-inflammatory and anti-diabetic activities of *Cicer arietinum* leaf aqueous extract (*CALAE*). *CALAE* showed antioxidant properties by scavenging DPPH free radicals in a dose-dependent manner with an IC_{50} value of 39.33µg/µl. The free radical quenching ability of *CALAE* was further evaluated using sodium nitrite and diclofenac induced oxidative stress in the RBCs and rat model. Interestingly, *CALAE* normalized stress markers such as Lipid peroxidation, protein carbonyl content, Total thiol, Superoxide Dismutase and Catalase in a dose-dependent manner in both RBCs and tissue homogenate of rats. Furthermore, the toxicity study of *Cicer arietinum* plant extract was done by direct hemolytic activity; data revealed that it did not lyse red blood cells; hence it is non-toxic in nature. Furthermore, *CALAE* inhibited denaturation of both egg and bovine serum albumin in a dose-dependent manner, suggesting its anti-inflammatory property. Interestingly, *CALAE* inhibition of the α-amylase and α-glucosidase activity showed the anti-diabetic property. In conclusion, *CALAE* showed, antioxidant, anti-inflammatory and anti-diabetic properties.

Keywords: *Cicer arietinum*, haemolytic activity, oxidative stress, anti-oxidant activity, anti-inflammatory activity

Introduction
Chickpea (*Cicer arietinum*) an edible legume belongs to the kingdom *Plantae*; division *Magnoliophyta*; class *Magnoliopsida*; order *Fabales*; family *Fabaceae* or *leguminosae*; subfamily *Faboideae* and genus *cicer* [1]. *Cicer arietinum* are grown in western Asia, Indian Subcontinent and Australia [2]. Most of the developing countries chickpea has been utilized as a staple food for humans. Although, chickpea seeds are predominantly consumed, the leaves are also cooked and consumed especially in India and Nepal. Chickpea leaves are richest source of minerals, vitamins, carbohydrates and secondary metabolites such as alkaloids, anthocyanins, catechins, fiber, flavonoids, phytic acid, quercetin, saponins, steroids, tannins, and terpenoids and trypsin inhibitors [3]. The said cocktails of phytochemicals present in the chickpea leaves responsible for numerous health benefits [4]. Leaves of chickpea exhibits anti-inflammatory, anti-diabetic as well as antioxidant properties [5]. Extensive research has been done on the biochemical, nutraceuticals characterization and pharmacological evaluation of chickpea seeds. While, their beneficial role on oxidative stress induced pathogenesis is least explored. Oxidative stress is the imbalance between endogenous antioxidants (enzymatic and non-enzymatic) in the cell and production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS/RNS at minimal level very much essential for the several physiological functions, while at the increased rate they triggered delirious effects such as inflammation and tissue damage [6]. The latter is the hallmark for the augmentation of diabetes, cancer, thrombosis, cardiovascular complications and neurodegenerative diseases. ROS and RNS are normally generated by tightly regulated enzymes, such as NO synthase (NOS) and NADPH oxidase isoforms, respectively [7]. Inflammation is a kind of body's immune system against irritants such as infection, injury, or harmful chemical. Free radicals progressively cause the inflammation in a complex manner resulting many human diseases [8]. Inflammation is of two types acute and chronic inflammation.

The free radicles such as ROS/RNS generated by the body poses various physiochemical and pathological conditions [9]. The progressive generation of oxidative stress causes chronic inflammation often led to cancer, diabetes, cardiovascular, neurological, and pulmonary diseases [10]. Diabetes mellites is a syndrome characterized by loss of glucose homeostasis. The disease is progressive and is associated with high risk of atherosclerosis, kidney and nerve damage as well as blindness [11]. New findings on the lack of antioxidant and vitamins as well as more oxidative damage are discussed in connection with Type 1 and Type 2 diabetes mellitus. Certain theories connect the changes in glutathione's redox potential and oxidative stress to both reactive oxygen species and hyperglycemia [12]. Many people are turning toward antioxidants since they help with antiaging and anti-inflammation [13]. The pre-radicals produced during metabolism are neutralized by molecules known as antioxidants [14]. Radicals are controlled by including vitamin C and vitamin E in our diet. Catalase and dismutase which our bodies naturally produce, help eliminate the radicals produced [15]. At present, adding antioxidants to foods and food products improves the quality of food and helps prevent it from being spoiled because of decay. Furthermore, antioxidants are often used in pharmacology, cosmetics and medicine, owing to their ability to reduce damage [16]. In this context, in the current study we made an attempt to evaluate the potential role of *Cicer* Aqueous Leaves Extract (CALAE) mitigating oxidative stress induced inflammation, tissue damage and diabetes.

Materials and Methods

Reagents

Methanol, DPPH (1,1-diphenyl-2-picrylhydrazyl), $NaNO_2$(Sodium Nitrate), EDTA (Ethylene diamine tetra acetic acid), TEMED (Tetra methyl ethylene diamine), DNPH (2,4-dinitro phenyl hydrazine), TCA(Tri chloro acetic acid), DTNB (5,5′-dithiobis-(2-nitrobenzoic acid)-Elman's reagent, SDS (Sodium dodecyl sulphate), Acetic acid, Thiobarbituric acid, H_2O_2 (Hydrogen peroxide), DNS (3,5 dinitro salicylic acid), NaOH (Sodium hydroxide), Sodium potassium tartarate, Declofenac, Starch, PBS (Phosphate buffer saline), BSA (Bovine serum albumin), Egg albumin, α-amylase, α-glucosidase, Trypsin and P-nitrophenyl-d-glucopyranoside,The human blood samples obtained from the healthy donors for the Platelet Rich Plasma (PRP).

Preparation of *Cicer* Aqueous Leaves Extract (CALE) and Protein estimation

Cicer arietinum leaves were collected from Komalapura village of Mysore. Leaves were milled into powder with a grinder (SHARP, Japan) and homogenized in double distilled water and centrifuged at 1500 g for 20 min at 15 °C. This extracted protein sample was used throughout the study. Protein concentration was assayed as described by Lowry *et al*., [17] using Bovine serum albumin (BSA) as standards.

In-vitro antioxidant activity

DPPH assay

The Antioxidant activity was measured by evaluating the *In vitro* fractions of free radical scavenging capability using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay. *CALAE* (0-100 µg) was used for the study and ascorbic acid was used as a positive control. The sample containing tubes was made up to 2.5 mL using methanol before being properly mixed into a 10 mM solution of DPPH in 50% methanol. 140 µL of 10 mM DPPH reagent was added and the mixture was mixed thoroughly and kept for incubation for 30 min at room temperature. After the incubation period, the absorbance was read at 517 nm using a UV-Vis spectrophotometer [18]. The percentage of DPPH inhibition was measured by calculating the% scavenging activity using below mentioned formula:

% scavenging activity = Control optical density-Sample optical density × 100 / Control optical density.

Oxidative stress induction using Sodium nitrite ($NaNO_2$)

The oxidative stress was induced using sodium nitrite in Red blood cells (RBC) model, drawn the blood from a healthy human being of age 22-35, and the donor was non-alcoholic, non-medicated, non-smoker and stress-free person. The blood drawn from the healthy donor and collected in presence of anticoagulant Acid Citrate Dextrose (ACD), centrifuged the blood for 13 min at 900 rpm, after the centrifugation, the blood was separated as plasma and RBCs in the form of supernatant and pellet respectively. Collected the RBCs and washed the cells using phosphate saline buffer for 3 times at 3500 rpm for 10 min on each time, prepared the 2% haematocrit using washed RBCs and phosphate saline buffer. For prepared haematocrit oxidative stress was induced by adding 10 mM $NaNO_2$ [19].

Lipid peroxidation (LPO) assay

Lipid peroxidation was determined using a previously published procedure [20]. Different concentrations of CALAE (0-100 µg) and erythrocyte lysate (0.02 mL; 2 mg of protein) make up the reaction mixture. It was incubated for 30 minutes at 26 °C after 10 mM NaNO2 caused the lipid peroxidation. Following the incubation period, add 1.5 mL of acetic acid (pH 3.5), 0.2 mL of 8% SDS, and 1.5 mL of TBA (0.8%) to the reaction mixture. The mixture was then incubated for 45 minutes at 45-60 °C, following the addition of butanol and pyridine in a 2:1 ratio. The absorbance at 530 nm was measured after the reaction was well mixed and centrifuged for 15 minutes at 3000 rpm.

Estimation of Protein carbonyl content (PCC)

Following a previously published procedure, DNPH was used to measure the protein's carbonyl content [21]. In short, 200 µL of RBC lysate (2 mg protein/mL) was mixed with $NaNO_2$ (10 mM) and CALAE (0-100 µg), and the combination was then incubated for 30 minutes. The combination was then incubated for an additional hour at room temperature with periodic shaking. An equivalent volume of RBC lysate (10 mM DNPH) was then added to a series of tubes containing tubes other than RBCs alone, and 2 N HCl was added to the RBCs alone. After incubation, 20% TCA was added to precipitate the mixture, and it was centrifuged for 10 minutes at 2000 rpm. After that, it was allowed to sit at room temperature for five minutes. The precipitate was dissolved in 1 mL of Tris buffer (20 mM pH 7.4 with 0.14 M NaCl, 2% SDS) after being thoroughly cleaned twice with acetone. The absorbance of the supernatant was then measured at 360 nm. The carbonyl

content was expressed as the number of carbonyl groups per milligramme of protein.

Measurement of Total thiols (TT)

Total thiols were determined using the previously reported technique [22]. CALAE (0-100 µg) was combined with 200 µL of RBC lysate (2 mg protein/mL) and treated with NaNO2 (10 mM). After 30 minutes of incubation, add 0.375 mL of 0.2 M Tris-HCl buffer (pH 8.2), 10 mM dithiol-bis-nitro benzoic acid (DTNB), and 1.975 mL of methanol, cover the reaction mixture, and incubate for another 30 minutes at room temperature. The tubes were centrifuged for 10 minutes at 5000 rpm. The clear supernatants from the samples were collected and their photometric absorbance at 412 nm was measured. The concentration of thiol in one mole of oxidised DTNB per milligramme of protein.

Superoxide dismutase (SOD)

The enzyme superoxide dismutase activity was measured followed by the previous method [23]. *CALAE* (0-100 µg) was incubated with an agonist, $NaNO_2$ (10 mmol/L), RBCs (2 mg of protein) in a clean, dry test tubes for 30 min at room temperature. Added 1 mL of the reaction mixture, which was made up of a combination of TEMED-EDTA (8 mmol/L) and phosphate buffer (PBS-16 mmol/L, pH 7.8). The decrease in absorbance was noticed at 406 nm for 1 min. U/mg of protein was used to express the results.

Catalase (CAT)

CAT enzyme activity was determined using a previously established method [24]. CALAE (0-100 µg) was given to RBCs (2 mg protein) in clean, dry test tubes, along with an agonist, sodium nitrite (10 mM), and incubated for 30 minutes at ambient temperature. To the reaction mixture, add 8.8 mM hydrogen peroxide and 100 mM sodium phosphate buffer (pH 7.4). The absorbance was measured at 240 nm for three minutes. The unit of measurement for CAT activity was U per mg of protein.

***In-vivo* studies on oxidative stress**

Animal grouping

The in-vivo study was conducted using a male Sprague Dawley rat as the animal model. The rats were purchased from the Tumkur, Karnataka, India-based Institution of Chromed Bioscience facilities and kept in polypropylene cages with six rodents each. Rats measuring 75-100 g and six-eight weeks of age were employed in the experiments. Male Sprague Dawley rats weighing 75 to 100 grams were put into seven groups of six animals each. A standard laboratory diet consisting of pellets was fed to the experimental animals, and they were allowed unlimited access to water. Each cage had 12 hours of light and dark cycles, a temperature range of 25-32 °C, and a humidity range of 55-65%. Under reference CBPL-IAEC-027-01/2023, the Institutional Animal Ethics Committee accepted the study design and experiment procedure. The following methodology was used:

Control group I (normal saline)

- **Group II: Diclofenac by itself**
- **Group III:** Received intraperitoneal injections of silymarin (25 mg/kg body weight/day) and diclofenac (50 mg/kg body weight/day) 45 minutes later.
- **Group IV:** Diclofenac (50 mg/kg body weight/day) was given 45 minutes after intraperitoneal injection of CALAE (50 mg/kg body weight/day).
- **Group V:** Diclofenac (50 mg/kg body weight/day) was given 45 minutes after intraperitoneal injection of CALAE (100 mg/kg body weight/day).
- **Group VI:** Received an intraperitoneal injection of CALAE (150 mg/kg body weight/day) followed by a 45-minute administration of 50 mg/kg body weight/day of diclofenac.
- **Group VII:** Received CALAE (150 mg/kg body weight/day) only.

After receiving medication for seven days, the rats were fasted for twelve hours. On the seventh day, the animals were put to sleep with diethyl ether, and blood samples were taken by heart punctures. Two millilitres of blood were drawn from the aorta using sterile, anticoagulant-free voiles in order to evaluate biochemical parameters. Alkaline phosphatase, albumin, globulin, total bilirubin (direct and indirect levels), SGOT, SGPT, and other biochemical markers were also measured. Before being homogenised for biochemical examination, the experimental animals' liver, kidney, pancreas, and heart were removed and kept in phosphate buffer saline solution. In order to facilitate histological analysis, the organs were also preserved in 10% formalin.

Histopathological Examination

The liver, kidney, pancreas, and heart samples from each group were processed and paraffin embedded for histological evaluation. Sections with thicknesses ranging from 3 to 5 µm were stained with hematoxylin and eosin stains.

Determination of *in-vivo* antioxidant activities

In order to analyse stress markers, tissue samples from the experimental rats' essential organs the liver, kidneys, heart, and pancreas were obtained using chilled ice-cold 0.1 M PBS. The tissues were then extensively homogenised.

***In-vitro* anti-inflammatory activity**

Egg albumin and Bovine serum albumin denaturation assay

The protein denaturation assay was followed by previously described method [25]. The 0.2 mL of 1% bovine albumin and egg albumin mixed with a *CALAE* (0-100 µg) and 4.78 mL PBS. Aspirin was used as a positive control. Incubated the reaction mixture for 15 min at room temperature before being heated in a water bath at 70 °C for 5 min. Cooled the tubes containing mixture and read the absorbance of the turbidity using spectrophotometer at 660 nm. The% of inhibition of denaturation was calculated using the formula:

% of inhibition of denaturation=1-Absorbance of test sample/ Absorbance of the control ×100

Protease Inhibitory activity

The method was used to measure the protease inhibitor [26]. The *CALAE* (0-100 µg) was added in a clean dry test tube with a mixture of 1 mL of 20 mM Tris-HCl buffer (pH 7.4) and 0.06 mg trypsin and incubated the reaction mixture for 5 min at room temperature. About 1 mL of 0.8% (w/v) casein was added and kept the reaction for 20 min. The reaction was stopped by 2 mL of 70% per chloric acid added to the

tubes contained reaction mixture. Centrifuged the tubes for 10 min at 1500 rpm. Collected the supernatant and absorbance was measured at 210 nm. The aspirin was used as a positive drug controller.% of inhibition of denaturation was calculated using the formula mentioned above in the protein denaturation.

In-vitro anti-diabetic activity

Inhibitory activity of α-amylase

The inhibitory activity of α-amylase was performed by following previously described method [27]. 250 mL of starch 2.0% (w/v), 250 mL of 1 U/mL α-amylase solution, and *CALAE* (0-100 µg) extract were mixed together in a test tube in a homogenous manner. Dinitrosalicylic acid 500 µL was added to the mixture to halt the enzymatic reaction. Then the reaction mixture was incubated at 200 ℃ for 3 min. 250 mL of 250 U/mL α-amylase was added right away to the mixture after it had been kept in boiling water. For 15 min, the mixture was heated. In addition, the solution was stopped from heating and cooled for 3 min at room temperature. A total volume of 6 mL was obtained by adding 4.5 mL water and with the aid of a vortex, the solution was homogenised. The absorbance at 540 nm was used to determine α-amylase activity. The following formula was used to calculate inhibition rates as percentage controls.

% of inhibition of α-amylase = Absorbance of control-Absorbance of sample/ Absorbance of control × 100

Inhibitory activity of α-glucosidase

The *in-vitro* Inhibitory activity of α-glucosidase was carried by following previously described method [28]. The synthesised *CALAE* were pre-mixed with α-glucosidase at various concentrations (0-100 µg) in 100 mL of ice-cold distilled water. P-nitrophenyl-d-glucopyranoside (3 mM) was added to the mixture as a substrate in potassium phosphate buffer to initiate the reaction. After 30 min at 37 °C, the reaction was stopped by adding Na_2CO_3 (2 mL, 0.1 M). The release of p-nitro phenyl-d-glucopyranoside at 420 nm was used to measure α-glucosidase activity. Inhibition rates were calculated as percentage controls using the following formula:

% of inhibition of α-glucosidase = Absorbance of control-Absorbance of sample/ Absorbance of control × 100

Results

CALAE via antioxidant potential ameliorates the oxidative stress parameters in RBC model (*In-vitro*)

The *CALAE* effectively scavenged di-Phenyl-2-picryl-Hdrazyl (DPPH) free radicals in a dose-dependent manner. The antioxidant potential of the *CALAE* was found to be 83.47% with the IC_{50} value 39.33 µg/µL, whereas the standard ascorbic acid (Vitamin C) was found to be inhibit 88.05% of generated radicals. (Figure1) illustrates the antioxidant activity of *CALAE*. Additionally, by assessing the $NaNO_2$-induced oxidative stress parameters in the RBC model (*In-vitro*) the antioxidant role was assessed by evaluating biomarkers include lipid peroxidation, protein carbonyl content, and total thiol content. Anti-oxidant enzymes like catalase and superoxide dismutase are utilized to analyze oxidative stress. Polyunsaturated fatty acids exposed to free radicals at an elevated level result in the formation of lipid peroxidation by generating products like Malondialdehyde (MDA), 4-hydroxy-2-nonenal and acroline. In our studies for the estimation of Lipid peroxidation, the amount of liberation of MDA was tested in the presence of a TBA mixture. Interestingly, our sample reduced the amount of MDA liberated in a dose-dependent manner. Additionally, protein carbonylation and thiolation were performed to determine the elevation levels of protein carbonyl content and total thiol content, respectively. The *CALAE* decreased the levels of PCC and TT in a dose-dependent manner as compared to the positive control ascorbic acid (Figure 2). Furthermore, the level of antioxidant enzymes was determined in a $NaNO_2$-induced oxidative stress RBCs model; during the level of stress overdose at that time, the activity of antioxidant enzymes decreased; hence, *CALAE* improved the activity of SOD and CAT and normalized the level of these enzymes. The (Figure 3) represents the activity of SOD and CAT against sodium nitrite-induced oxidative stress.

CALAE ameliorates the diclofenac induced oxidative stress parameter in animal model (*In-vivo*)

The levels of PCC, MDA, and TT were considerably higher in the rat liver, kidney, pancreas, and heart homogenates that had been injected with 50 mg/kg of diclofenac than in the positive control silymarin. Rats with 150 mg/kg of CALAE showed significantly decreased levels of MDA, PCC, and TT in their liver, kidney, pancreas, and heart homogenates when compared to the control groups. Compared to control groups, rat homogenates treated with CALAE alone showed normal levels of MDA, PCC, and TT (Figure 4). Endogenous antioxidant enzymes (CAT and SOD) were significantly reduced in rat sample homogenates treated with DFC. Antioxidant enzyme activity was restored in the liver, kidney, pancreatic, and heart homogenates of rats given DFC after they had been pre-incubated with CALAE (150 mg/kg). SOD and CAT activity were unchanged as compared to the CALAE-alone-treated animals (Figure 5).

CALAE Restore the biochemical parameters *in-vivo*

The biochemical markers, including albumin, globulin, and total protein levels, were reduced in the blood samples from the rats treated with DFC. However, there was a considerable increase in bilirubin, SGPT (serum glutamate pyruvate transaminase), alkaline phosphatase, total bilirubin, indirect bilirubin, and directs of SGOT (serum glutamate oxaloacetate Trans aminase). In contrast to the experimental animals' positive control silymarin-treated group, the levels of the aforementioned biochemical parameters returned to normal after DFC pre-incubation with the CALAE injected group (Figure 6).

CALAE restore the liver, kidney, heart, and pancreas morphology

In contrast to the positive control treatment group of animals (Figure 7A3, B3, C3&D3), microscopic analysis of the liver, kidney, heart, and pancreas from the control group (Figure 7 A1, B1, C1, D1) and the CALAE alone treated group (Figure 7A6, B6, C6, & D6) revealed no abnormalities of tissue morphology. However, it was shown that the diclofenac-treated rats had severe liver, kidney, heart, and pancreatic tissue deterioration (Figure 7A2, B2, C2&D2). Increased cell infiltration in liver histology revealed

hepatocellular deterioration. The kidneys have tubular degeneration. Gallstone injury was seen in the pancreatic muscle, and heart muscle necrosis was seen in the heart. It's interesting to note that CALAE, in a dose-dependent manner, repaired the damaged liver, kidney, heart, and pancreas from oxidative stress caused by diclofenac (Figure 7,A4,5,6,B4,5,6, C4,5,6, & D4,5,6).

CALAE exhibits anti-inflammatory active

CALAE decreased RBC membrane stabilisation in a dose-dependent manner and shown strong anti-hemolytic efficacy when compared to aspirin, a positive control (Figure 8A). Furthermore, by reducing protein denaturation in a dose-dependent way, CALAE prevented the denaturation of egg albumin and bovine serum albumin and had an anti-inflammatory impact. CALAE exhibited the highest denaturation inhibition when compared to aspirin (90%) as well. About 80% inhibition of eggs albumin and 75% inhibition of bovine serum albumin were observed (Figure 8B&C). Additionally, the inhibitory activity of serine protease was used to further analyze the anti-inflammatory impact. Interestingly, CALAE decreased trypsin activity significantly in a concentration-dependent manner when compared to positive control aspirin. It was discovered that aspirin inhibited trypsin by 80% whereas CALAE inhibited it by 94%.

CALAE showed _In-vitro_ anti-diabetic activity

CALAE was inhibited α-amylase and α-glucosidase activity in a dose dependent manner. The inhibition percentage was found to 70% in case of α-amylase and 72% for α-glucosidase respectively (Figure 9). The observed α-amylase and α-glucosidase inhibitory activity of *CALAE* was compared with the positive control acarbose (90%).

Toxicity effect of _CALAE_

CALAE does not lyse the RBCs as determined by direct hemolytic assay and the result reveals the non-toxic nature of *CALAE* on RBCs model (Figure 10).

Discussion

Oxidative stress mainly affects the cellular functions through the increased production of ROS/free radicals as there is a massive alteration of biological molecules such as proteins, lipids and DNA. Thus, oxidative stress has been considered as the key culprit in the pathogenesis of inflammation, diabetes, thrombosis [29]. Due to the life-threatening side effects triggered by currently available drugs which have been used against oxidative stress induced diseases, researchers looking for an alternative therapy derived from medicinal plants. Plants with medicinal properties play an increasingly important role in the food and pharmaceutical industries for their functions in disease prevention and treatment [30]. *Cicer arietinum* is a genus of the legume family *Fabaceae*. The common name of the *Cicer arietinum* is chickpea and it's native to Middle East Asia. Despite several medicinal qualities, the role of *Cicer arietinum* leaves phytochemicals on oxidative stress induced inflammation and diabetes is least studied so far. Thus, the current study we for the first time examined the role of aqueous extract of *CALAE* on regulating $NaNO_2$ induced oxidative stress along with anti-inflammatory and anti-diabetic potential. *CALAE* showed antioxidant properties by scavenging DPPH free radicals in a dose-dependent manner with an IC_{50} value of 75.21 µg/µl. The free radical quenching ability of *CALAE* was further evaluated using sodium nitrite-induced oxidative stress in the RBCs model. The measurement of oxidative stress has been quantifying the products of lipid peroxidation, protein carbonylation, thiolation [31]. The oxidation products produced from the reaction between oxygen and unsaturated lipids generates secondary products of Lipid peroxidation such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), propanol and hexanal. While 4-HNE is the most hazardous by-product of lipid peroxidation, MDA seems to be the most mutagenic [32]. Because of its simple reaction with thiobarbituric acid (TBA), MDA has been utilized extensively for many years as a handy biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids [33]. However, the protein carbonylation is a frequent oxidative alteration that can lead to structural or functional loss since it can cause aggregation, polymerization, unfolding, and conformational changes. Aggregates of oxidized protein build up and damage cells. Most involve carbonyls found in proteins [34]. Lysine, cysteine, and histidine are carbonylated when they react with reactive carbonyl groups that are created when lipids (like HNE, MDA, or acrolein (ACR)) and carbohydrates (like glyoxal (GO) and methylglyoxal (MGO)) are oxidised. This process of producing carbonyl is called lipoxidation and glycoxidation, which results in the creation of advanced glycation end-products (AGEs) [35]. The number of carbonyls in particular proteins can be ascertained by derivatising the carbonyl group with dinitrophenylhydrazine (DNPH), which produces a stable dinitrophenylhydrazone (DNP) product that can be examined spectrometrically or by immunoblotting [36]. Compared to other amino acids, cysteine residues are extremely infrequent in proteins, accounting for only 2.3% of the human proteome [37]. A variety of post-translational oxidative changes result from reactions between ROS or RNS species and the thiol (sometimes referred to as mercaptan or sulfhydryl)-SH side chain of cysteine [38]. The capacity of thiols in a sample to chemically decrease Ellman's reagent 5, 5′-dithiobis-(2-nitrobenzoic acid) or DTNB, which has a reactive disulfide bond that can be reduced. In a stoichiometric reaction, DTNB is reduced by free thiols via an exchange reaction that produces a mixed disulfide and a yellow-coloured 5-Thio-2-NitroBenzoic acid (TNB) [39]. *CALAE* significantly reduced the level of oxidative stress by reducing the markers like LPO, PCC and TT in sodium nitrite induced RBC damage. Over the previous reports documented some medicinal plants (Kenaf seed, Pomegranate leaves) showed good effect on antioxidant, anti-inflammatory, anticancer and antimicrobial activities [40]. Furthermore, our study also reports the *CALAE* normalized the level of endogenous antioxidant enzymes SOD and CAT activity in both sodium nitrites (RBCs) induced stress model.

Higher levels of ROS/RNS have been shown to trigger the inflammatory process by generating more pro-inflammatory cytokines, such as nuclear protein kappa B/active protein −1 and tumour necrosis factor alpha, which are linked to a number of chronic illnesses, including cancer [41]. The membranes of lysosomes and red blood cells are comparable. Through the inhibition of neutrophil release in particular bacterial proteins and proteases that cause damage upon extracellular discharge, leading to both acute and chronic inflammation, the stabilisation of the lysosomal

membrane plays a crucial role in the inflammatory response [42]. Inflammation-induced tissue damage and arthritic reactions are linked to protein denaturation [43]. Our findings illustrate the ability to regulate the protein denaturation involved in the inflammatory process by demonstrating that CALAE was successful in preventing the denaturation of protein (egg and bovine serum albumin) at all tested levels. Serine protease is the main enzyme secreted by neutrophils during inflammation, and it contributes significantly to tissue damage throughout the inflammatory response. In contrast to the positive control aspirin, *Cicer arietinum* inhibited trypsin, confirming its suspected anti-inflammatory properties. Newer pharmacological drugs were developed with the aid of recent developments in our understanding of intestinal enzyme function [44]. By blocking the activity of α-glucosidase at the intestinal brush border, α-glucosidase inhibitors decrease intestinal absorption of starch, dextrin, and disaccharides. By inhibiting this enzyme, postprandial glucose increase (PP hyperglycemia) is slowed and the absorption of carbohydrates from the GI tract is slowed. Insulin resistance and glycaemic index management in diabetics may benefit from this delay in starch digestion and breakdown [45]. As an α-glucosidase inhibitor, acarbose inhibits the absorption of complex carbs from the gut and decreases their digestion [46]. These medications may also lower blood sugar levels by increasing the release of the glucoregulatory hormone glucagon-like peptide-1 into the bloodstream [47]. However, they may lead to adverse effects such as malabsorption, stomach pain, gas, and diarrhoea, resulting in a high discontinuation rate [48]. Individuals with renal impairment should not be taken acarbose or miglitol. Acarbose should be taken with caution in patients with hepatic illness because it can produce reversible hepatic enzyme increase [49]. CLAE aqueous extracts inhibited α-glucosidase and α-amylase enzymes considerably, according to experiments.

Figure legends

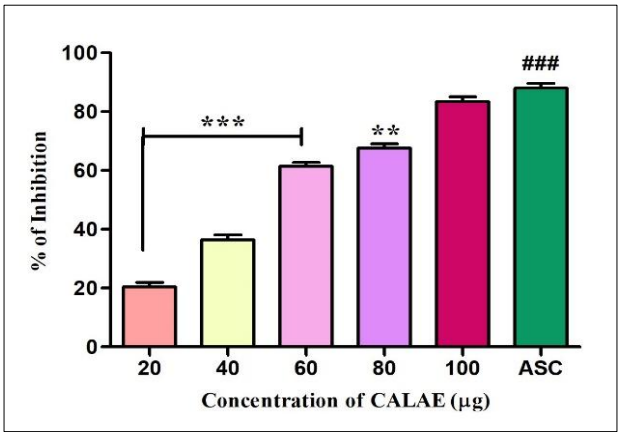


Fig 1: Antioxidant activity of *CALAE:* the DPPH free radical scavenging potential of *CALAE* examined by measuring its antioxidant activity in comparison with ascorbic acid. Each value is given as a mean ± SD.* Significance at $p<0.005$ and *** at $p<0.001$.

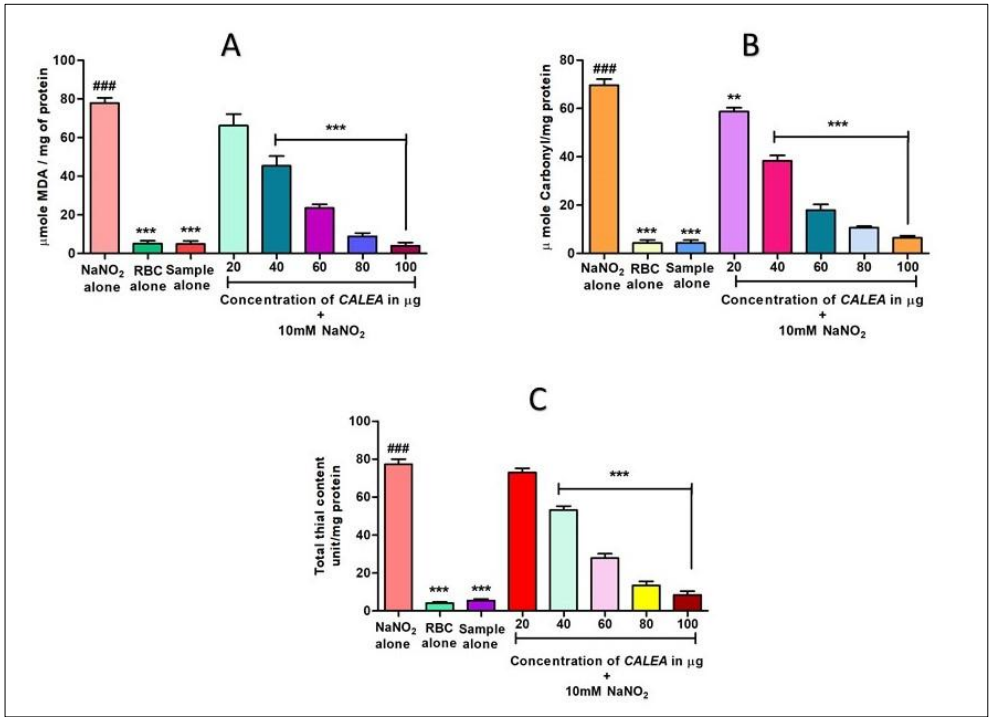


Fig 2: Role of *CALAE* on $NaNO_2$-induced oxidative stress in red blood cells: (A) lipid peroxidation, (B) protein carbonyl content and (C) total thiol content. To identify the protective effect of *CALAE* against $NaNO_2$-induced oxidative damage, red blood cells were pre-incubated for 10 min with various concentrations (50–150 µg/mL) of *CALAE* at 37 °C before treatment with $NaNO_2$ (10 mM). The results are presented in average units/mg of protein and are expressed as mean ± SEM (N=3). Significance at $p<0.005$, ** at $p<0.001$ and *** at $p<0.0001$.

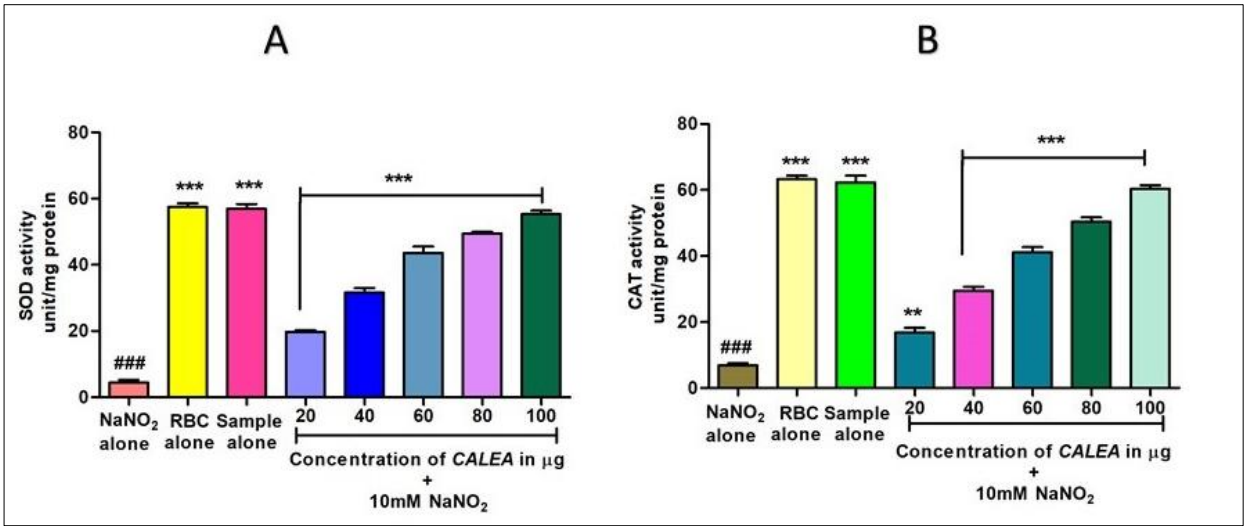


Fig 3: Role of *CALAE* on $NaNO_2$-induced oxidative stress in red blood cells: (A) Superoxide dismutase activity, (B) Catalase: To identify the protective effect of *CALAE* against $NaNO_2$-induced oxidative damage, red blood cells were pre-incubated for 10 min with various concentrations (50–150 µg/mL) of *CALAE* at 37 °C before treatment with $NaNO_2$ (10 mM). The results are presented in average units/mg of protein and are expressed as mean ± SEM (N=3). Significance at $p<0.005$, ** at $p<0.001$ and *** at $p<0.0001$.

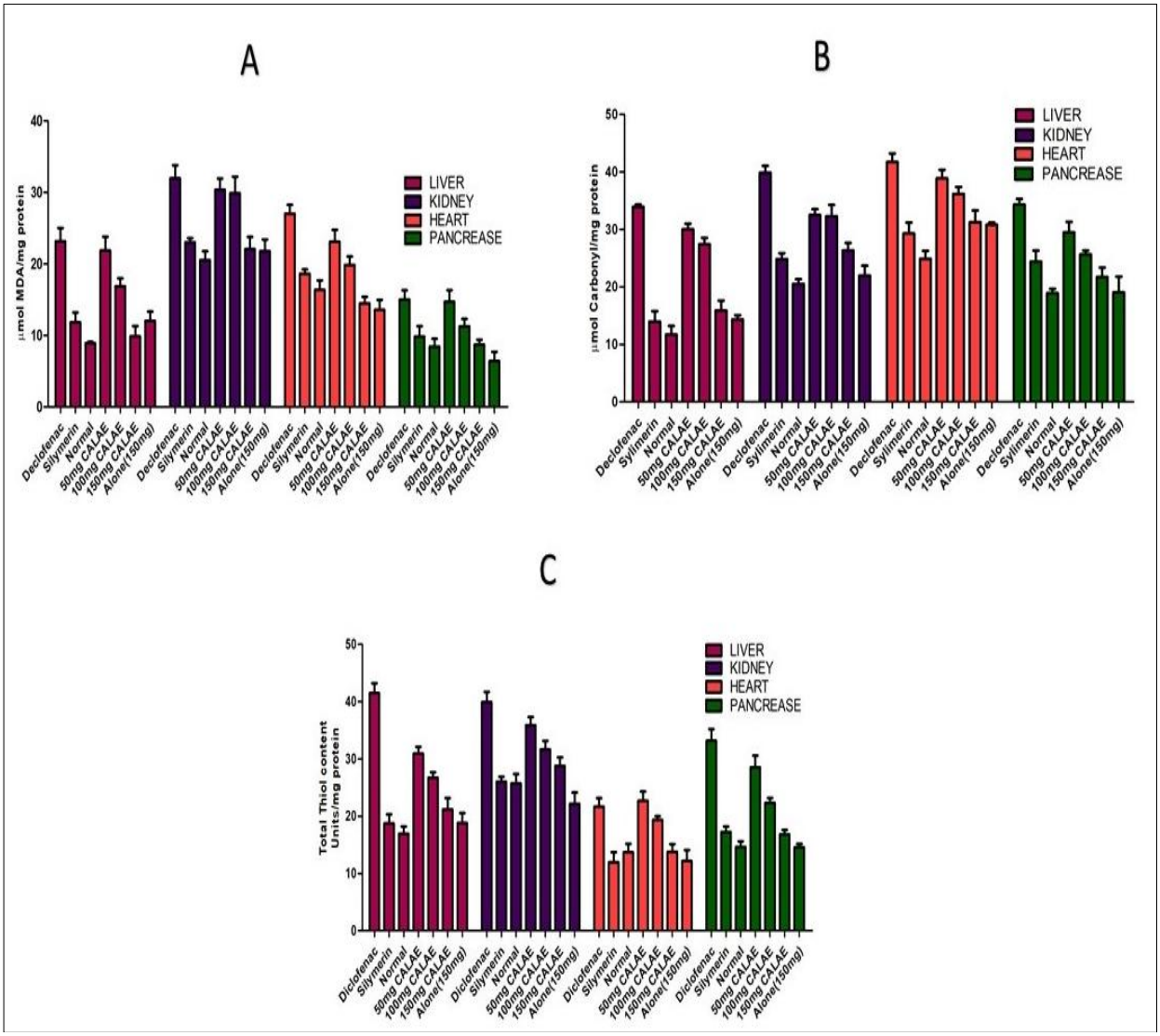


Fig 4: Effect of *CALAE* on oxidative stress induced by diclofenac in liver, kidney, heart and Pancreas: (A) Lipid peroxidation (LPO), (B) Protein carbonyl content (PCC), (C) Total thiol (TT). Control; Diclofenac (50 mg/kg); Silymarin (25 mg/kg) + Diclofenac (50 mg/kg); *CALAE* (50 mg/kg) + Diclofenac (50 mg/kg); *CALAE* (100 mg/kg) + Diclofenac (50 mg/kg); *CALAE* (150 mg/kg) + Diclofenac (50 mg/kg); *CALAE* alone (150 mg/kg) was administered according to the mg/kg bodyweight/day. All the samples were compared to the toxicity for control group. The data were represented as mean (n=3) ± SEM. **Significance at $p<0.001$ and *** at $p<0.0001$.

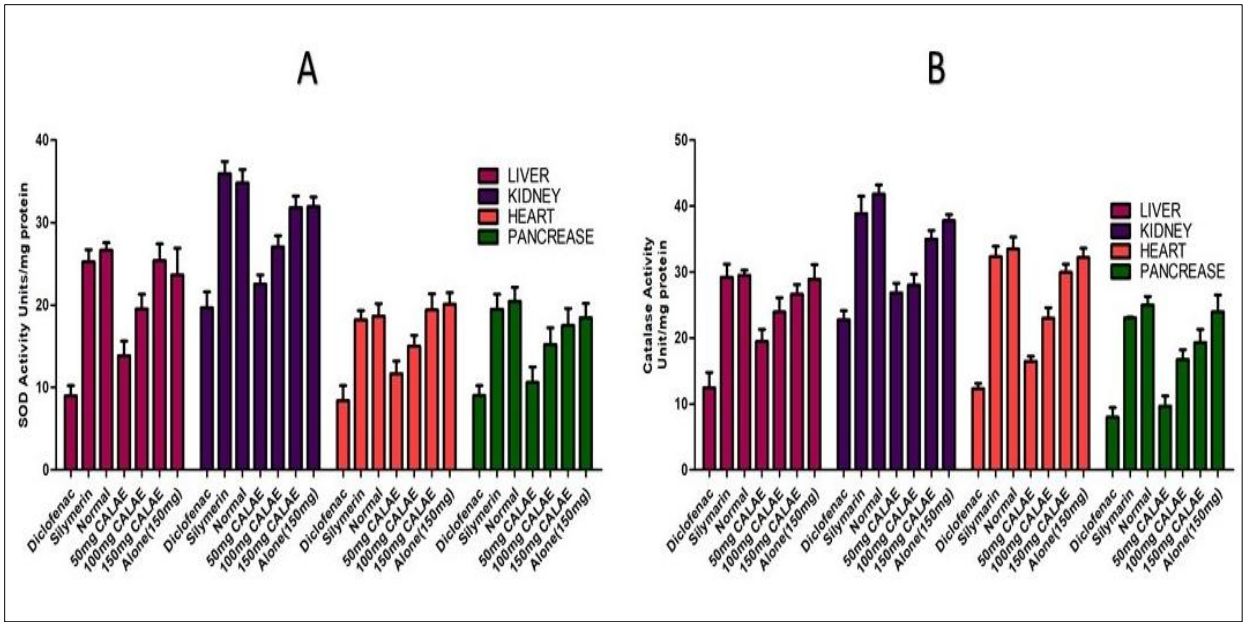
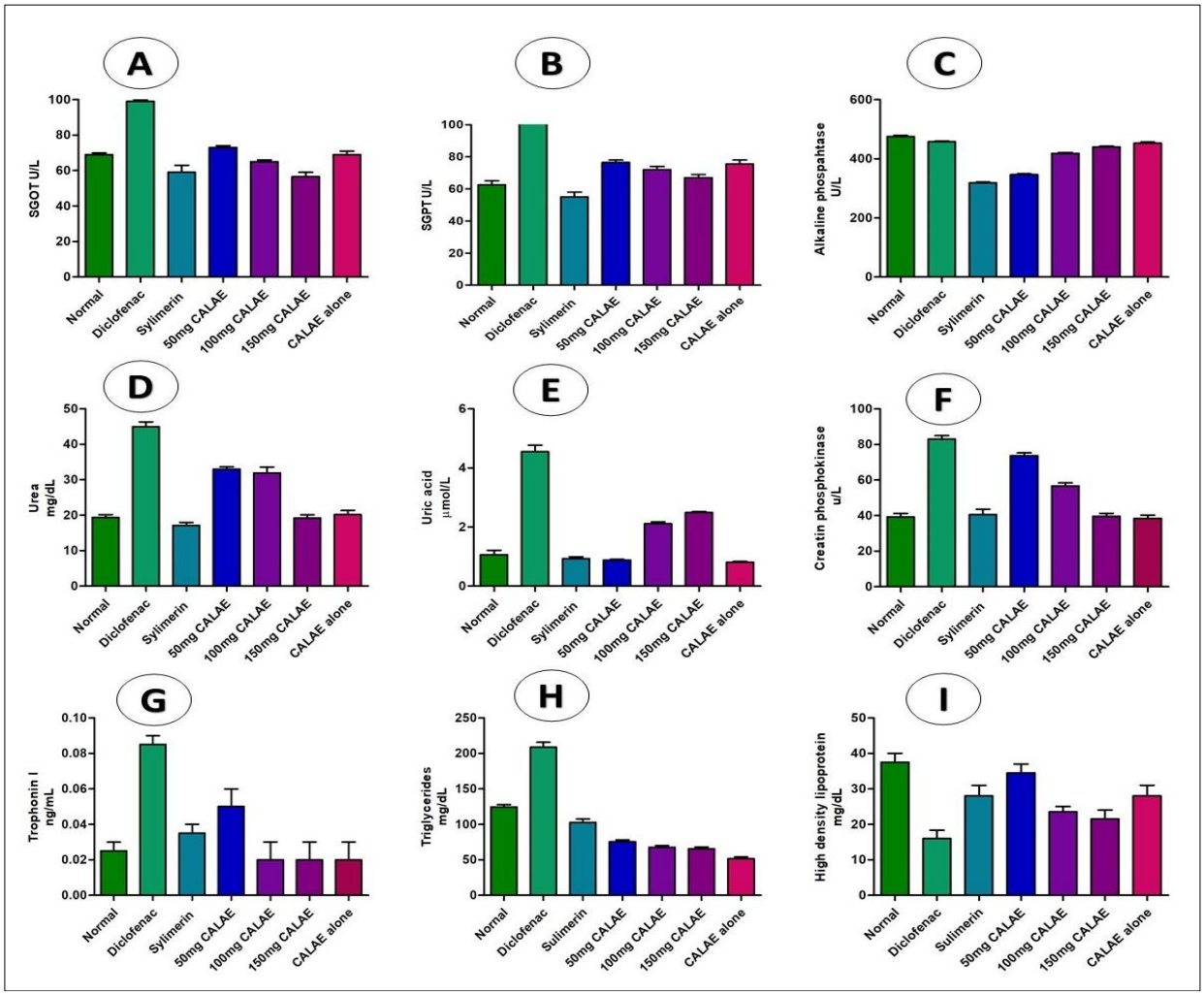


Fig 5: Effect of *CALAE* on oxidative stress induced by diclofenac in liver, kidney, heart and Pancreas: (A) Superoxide dismutase (SOD), (B) Catalase (CAT). Control; Diclofenac (50 mg/kg); Silymarin (25 mg/kg) + Diclofenac (50 mg/kg); *CALAE* (50 mg/kg) + Diclofenac (50 mg/kg); *CALAE* (100 mg/kg) + Diclofenac (50 mg/kg); *CALAE* (150 mg/kg) + Diclofenac (50 mg/kg); *CALAE* alone (150 mg/kg) was administered according to the mg/kg bodyweight/day. All the samples were compared to the toxicity for control group. The data were represented as mean (n=3) ± SEM. **Significance at $p<0.001$



and *** at $p<0.0001$

Fig 6: Serum biochemical marker analysis of Liver, Kidney, Heart and Pancreas: Liver Biochemical parameters such as SGOT, SGPT, ALP was shown in A, B, C. Kidney Biochemical parameters like Urea and Uric acid are shown in D and E. Heart biochemical parameters such as CPK and Troponin I are shown in F and G. Pancreatic biochemical markers such as Triglycerides and high-density lipoprotein were shown in H and I. All the data were expressed in SEM (0.002) with three trial replications.

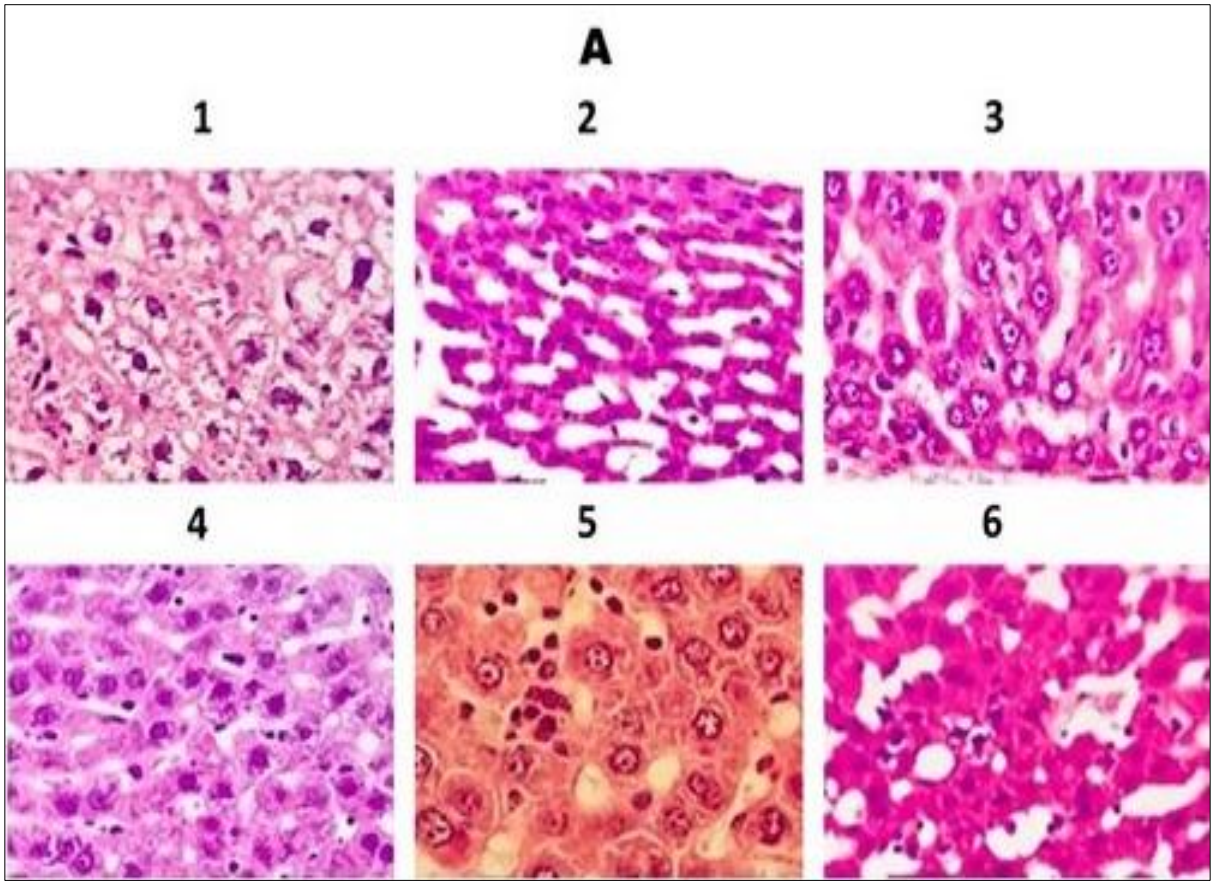


Fig 7: Effect of *CALE* on histology of liver, Kidney, Heart, and Pancreas: (A1) Normal liver tissue showing the control group; (A2) Diclofenac administered (50 mg/kg) group showed vacuolar changes; (A3) Diclofenac (50 mg/kg) preincubated with SYL administered (25 mg/kg) group showed mild vascular changes; (A4) Diclofenac (50 mg/kg) pre-incubated with *CALE* (50 mg/kg) restored to normal architectures; (A5) Diclofenac (50 mg/kg) pre-incubated with *CALE* (150 mg/kg) group showed almost normal liver histology; (A6) *CALE* alone (150 mg/kg) injected group showed normal liver histology.

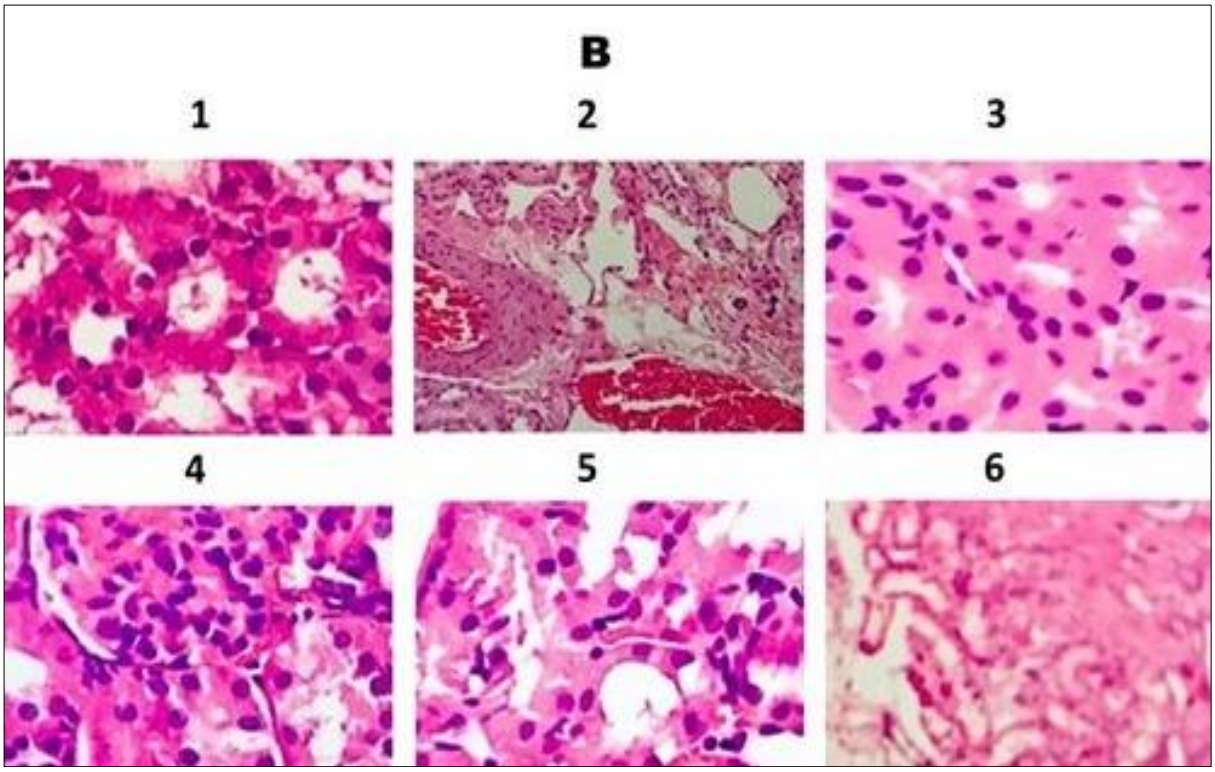


Fig 7: (B) Effect of *CALE* on kidney histopathology: (B1) Normal kidney tissue showing the control group; (B2) Diclofenac-administered (50 mg/kg)group showed tubular cell necrosis; (B3) Diclofenac (50 mg/kg) pre-incubated with SLY-injected (25 mg/kg)group showed mild tubular cell necrosis; (B4) Diclofenac (50 mg/kg) pre-incubated with *CALE* (50 mg/kg) restored normal architecture; (B5) Diclofenac (50 mg/kg) pre-incubated with *CALE*-injected(150 mg/kg)group restored almost normal architecture; (B6) *CALE*-alone-injected (100 mg/kg) group showed normal kidney histology.

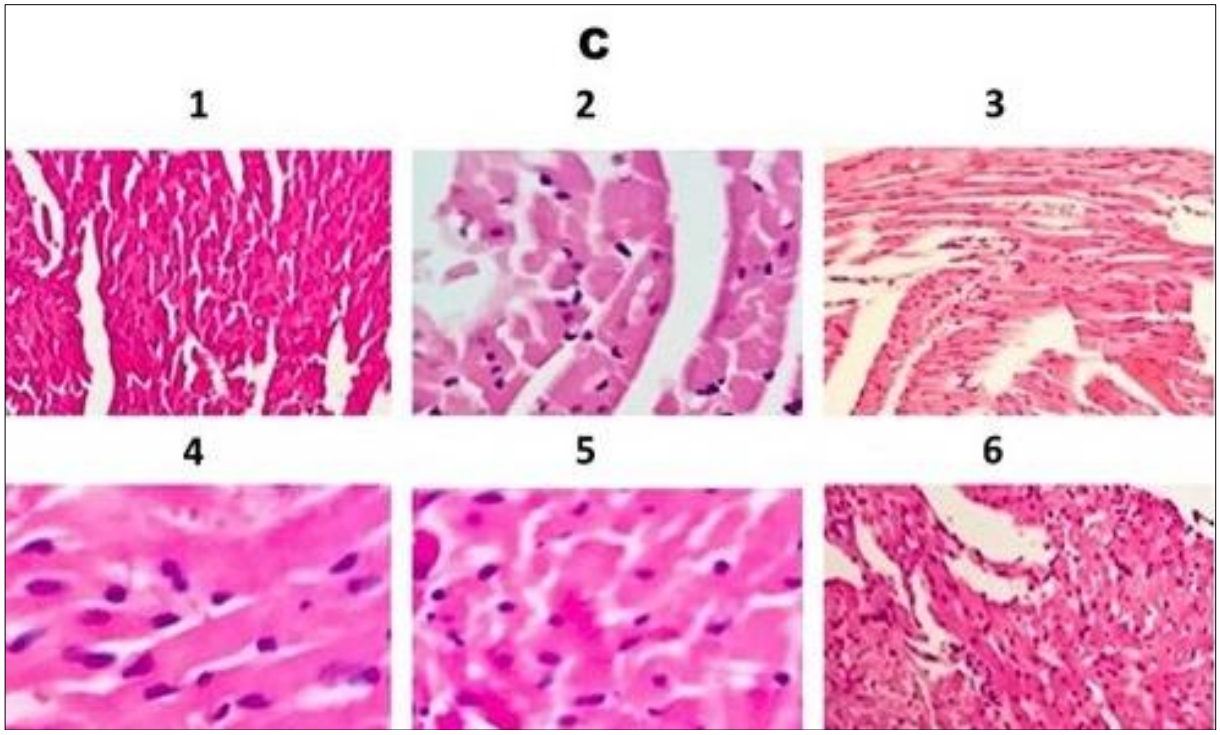


Fig 7: (C) Effect of *CALE* on the histopathology of heart: (C1) Normal heart tissue showing the control group; (B2) Diclofenac-administered (50 mg/kg)group showed cardiac cell necrosis; (B3) Diclofenac (50 mg/kg) pre-incubated with SLY-injected (25 mg/kg)group showed mild cardiac cell necrosis; (B4) Diclofenac (50 mg/kg) pre-incubated with *CALE* (50 mg/kg) restored normal architecture; (B5) Diclofenac (50 mg/kg) pre-incubated with *CALE*-injected(150 mg/kg)group restored almost normal architecture; (B6) *CALE*-alone-injected (100 mg/kg) group showed normal heart histology.

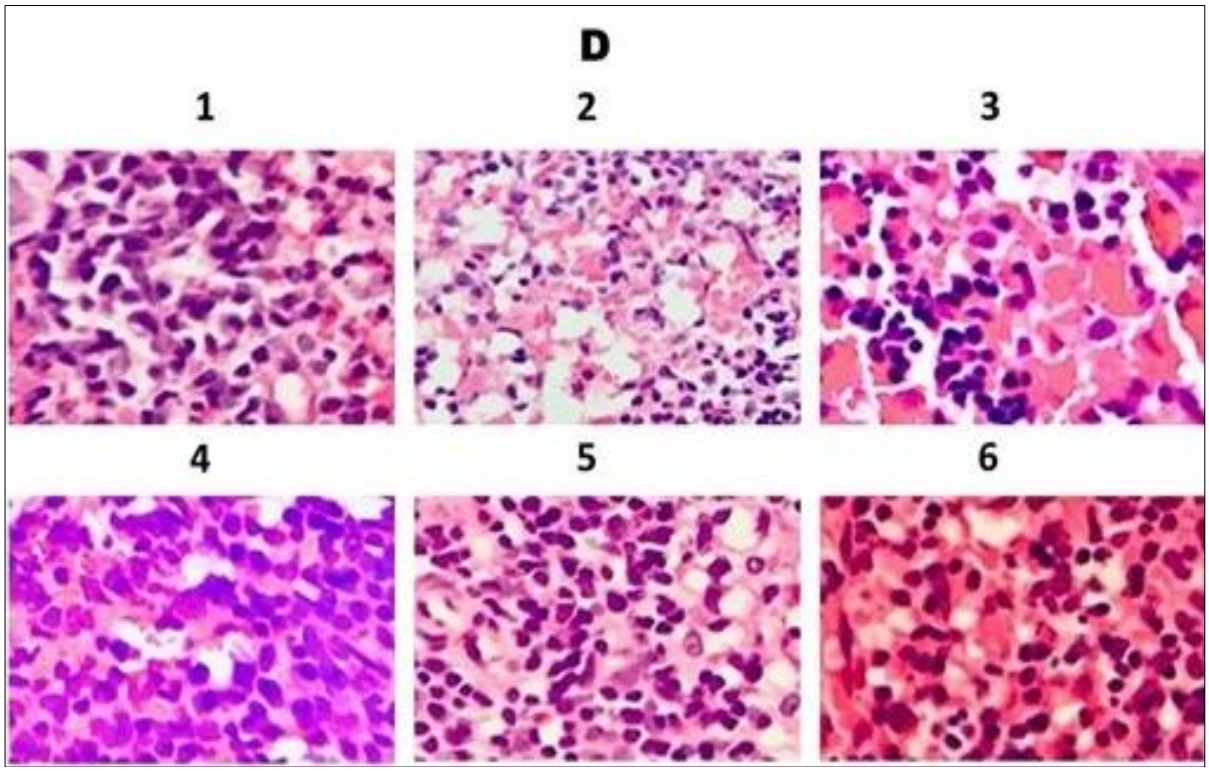


Fig 7: (D) Effect of *CALE* on the histopathology of pancreas: (D1) Normal pancreas tissue showing the control group; (D2) Diclofenac-administered (50 mg/kg)group showed islet of Langerhans cell necrosis; (D3) Diclofenac (50 mg/kg) pre-incubated with SLY-injected (25 mg/kg)group showed mild islet of Langerhans cell necrosis; (D4) Diclofenac (50 mg/kg) pre-incubated with *CALE* (50 mg/kg) restored normal architecture; (D5) Diclofenac (50 mg/kg) pre-incubated with *CALE*-injected(150 mg/kg)group restored almost normal architecture; (D6) *CALE*-alone-injected (100 mg/kg) group showed normal pancreas histology.

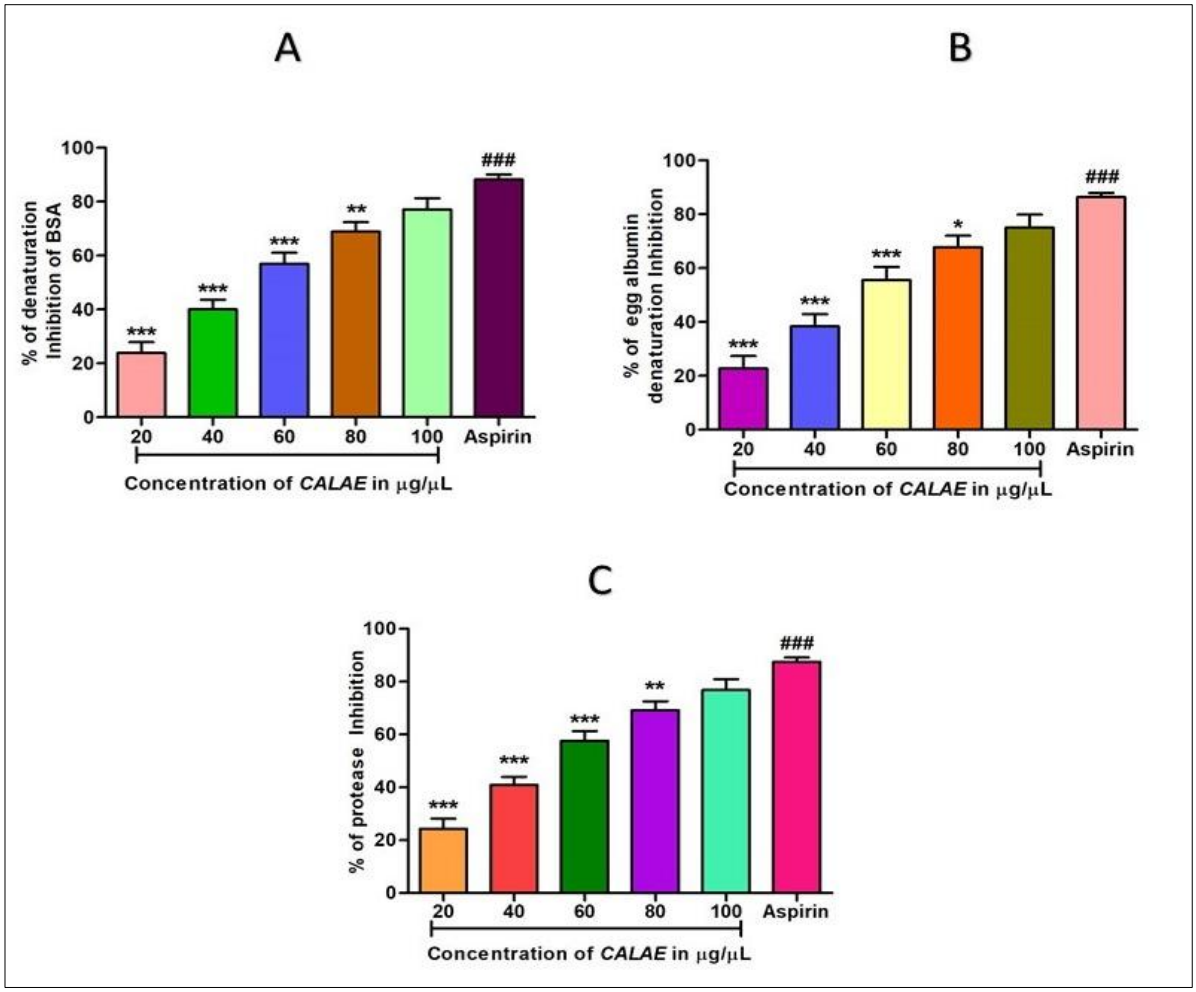


Fig 8: Effect of *CALAE* on protein denaturation: (A)% inhibition of BSA denaturation, (B)% inhibition of egg albumin denaturation, and (C)% of proteinase inhibition. The results are presented in average units/mg of protein and are expressed as mean ± SEM (N=3). * Significance at p< 0.005, ** at *p*<0.001 and *** at *p*<0.0001.

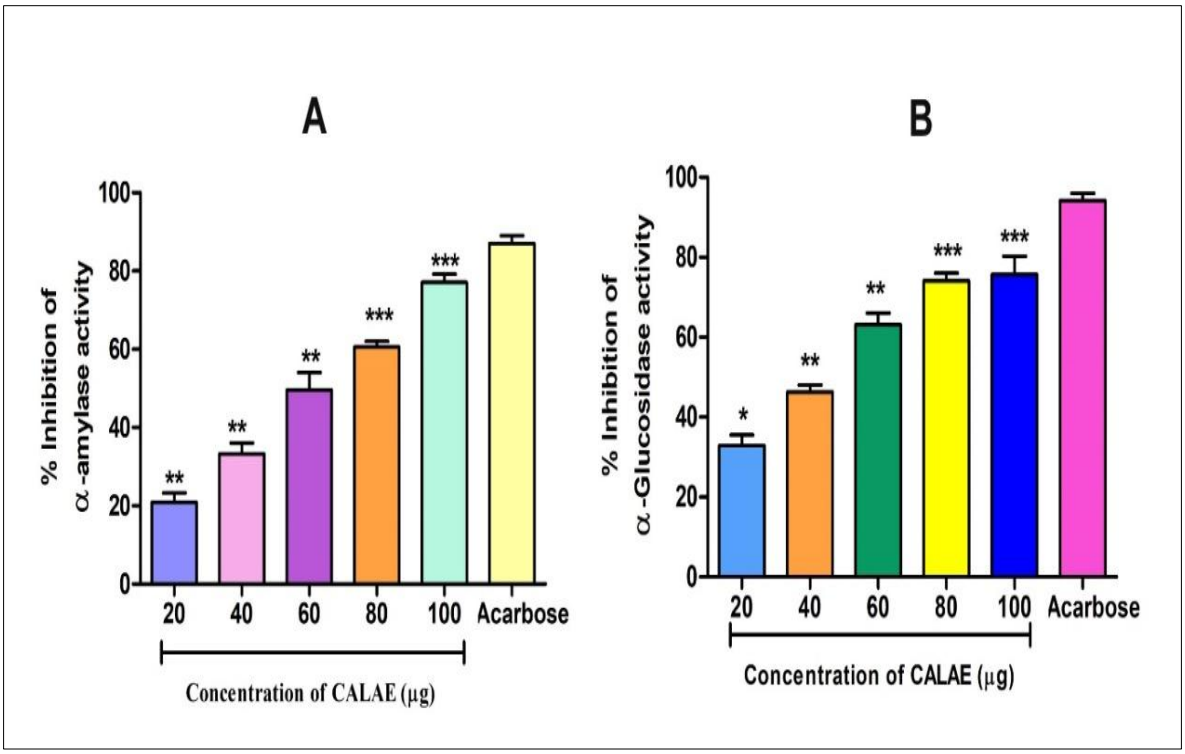


Fig 9: Effect of *CALAE* on Antidiabetic activity: (A)% inhibition of α-amylase activity, (B)% inhibition of α-Glucosidase activity. The results are presented in average and are expressed as mean ± SEM (N=3). * Significance at *p*≤0.005, ** at *p*<0.001 and *** at *p*<0.0001.

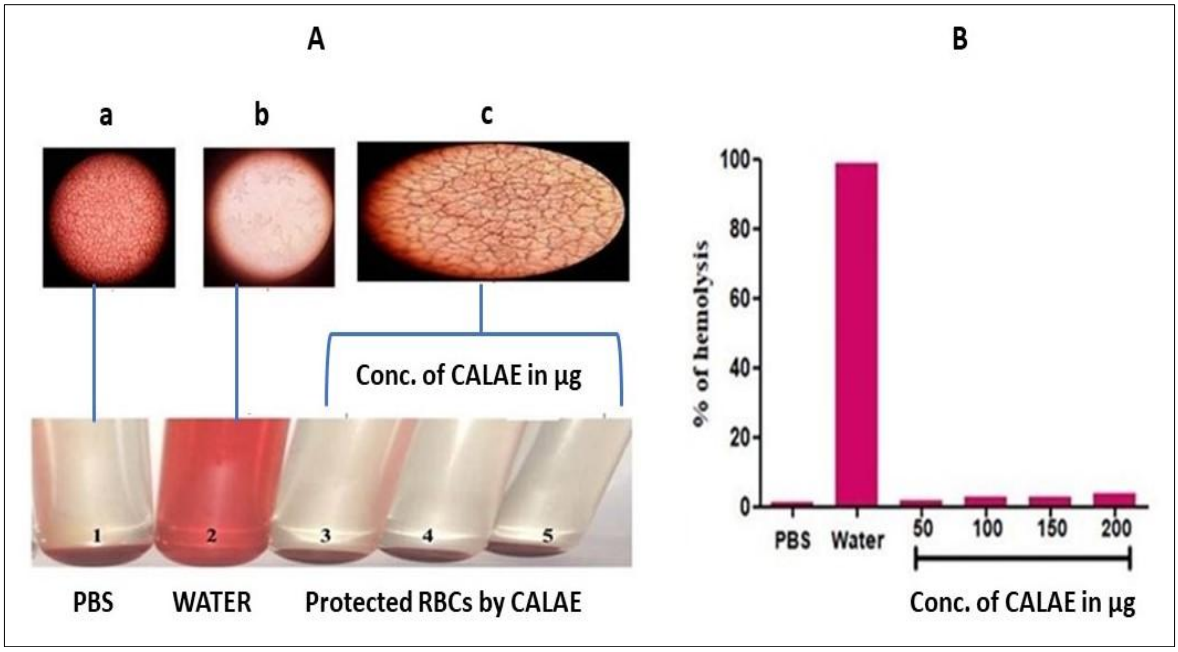


Fig 10: (A) Direct Hemolytic assay of *CALAE:* The different concentration of *CALAE* (50-150 µg) was incubated independently for 1h at 37 ℃ with the 1ml of suspension made with packed human erythrocytes and phosphate buffered saline (PBS) 1:9 v/v and view the non-hemolyzed RBCs viewed under microscope. (B) The amount of hemoglobin released in the supernatant was measured at 540snm and percentage of hemolysis was calculated

Conclusion

Cicer arietinum leaf aqueous extract *(CALAE*) was found to be enriched with secondary metabolites, and found to be potential antioxidant activities, that impart several bio-chemical activities by reducing the elevated stress in red blood cells, and also found to be re-established the biological anti-oxidant parameters *in-vivo*, and also found to decrease the level of inflammation and reduced the glucose levels released by maltose by enzyme action. So this chick pea plant will serve as a good candidate for resolving the above said related disorders.

Institutional review board statement

The animal experimental study was conducted following the Declaration and was approved by the Institutional Review Board (Ethics Committee Chromed bioscience, Tumkur protocol code-CBPL-IAEC-027-01/2023 and date of approvl-10-06-2023. The ethical approval was not applicable as no human samples were used in this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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Author contribution

Ramesh K Lakshmaiah-data analysis, Manjula M Venkatappa-experimental work, Shivakumar Venkataramaiah-supporting, Rajesh rangappa-resources, Devaraja Sannaningaiah-supervission and data correction.

Conflicts of Interest

The authors declare no conflict of interest.

Sample Availability

Samples available from the authors.

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