Chakraborty et al. Clinical Phytoscience (2018) 4:10
https://doi.org/10.1186/s40816-018-0067-x

Clinical Phytoscience

ORIGINAL CONTRIBUTION Open Access

A dose Dependent hepatoprotective and nephroprotective activity of eucalyptus oil on Streptozotocin induced diabetic mice model

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Abstract

Background: *Eucalyptus globulus* are a prime source of global eucalyptus oil production. This oil has therapeutic, flavoring, antimicrobial, and biopesticide properties. This oil has got popularity because they are reported as pharmacologically active.
A study has been conducted with the aim to find dose dependant effect of eucalyptus oil on diabetic mice induced with Streptozotocin (STZ) and analyze the hepatoprotective and nephroprotective nature through biochemical and histological study.
STZ at low dose was used to damage pancreatic β cells and induce type II diabetes on mice model. Oil was used in different percentages namely, 0.5%, 1%, 1.5%, 2% for treating hyperglycemia. Then after a treatment of 28 days, the total body weight, and biochemical parameters including blood glucose, serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), Bilirubin, cholesterol, urea, creatinine were checked thoroughly. A complete histological study of different organs like liver, kidney, and pancreas was done.

Results: From the explained experiment the result was quite interesting which showed among all the concentration of essential oil, 1.5% showed maximum effect on hyperglycemia with a rapid approach to normalize high blood glucose level and also other biochemical parameters. The tissue was got rejuvenated and behaved as normal cells after being treated with 1.5% oil.

Conclusion: So the main purpose of the work is to search for an alternative substituent of antidiabetic drug which can work as potentially as conventional drug for diabetes but without having side effects. And Eucalyptus oil proved to be safe and steady source for hyperglycemic patients at a particular dose. So it can be used for further diabetic research.

Keywords: Diabetes, Streptozotocin, Essential oil, Liver, Biochemical assay, drug dose dependant

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Background

Diabetes mellitus is a metabolic disorder, symptomised by hyperglycemia. This disease is the result of a deficiency in insulin secretion or insulin resistivity of the body [1].. They disturb the whole endocrine and metabolic balance of the body. According to American Diabetic Association patients having Type II diabetes are also susceptible to cardiovascular disease followed by renal failure, atherosclerosis, gastrointestinal disturbance, eye, skin, nerve complication.

STZ is a naturally occurring nitrosourea product. It is obtained from *Streptomyces achromogenes*. Lower dose (may be single dose) can cause severe toxicity to β cells in the pancreas which cause necrosis and then hyperglycemia occurs [2].

Mice model is mostly used in those experiments where lower dose of STZ generally used to induce type II diabetes and experimental drugs are applied [3].. In the present scenario different conventional marketised antidiabetic drugs like sulfonylurea, glucoside inhibitors, metformin, GLP-I agonist, troglitazones which are hypoglycemic in nature having immense side effects and not suitable for pregnants [4]. Some of the common side effects of these drugs are lower blood sugar level, weight gain, stomach upset, diarrhea, liver diseases, anemia etc. [5].

Thus, from this point of view scientific interest is now booming throughout the world in the study of medicinal plants, as plants are safe to live cells, having prolonged effect as well as due to lesser side-effects in clinical practice and relatively low costs in their treatment [6].

Many species of the genus *Eucalyptus* belonging to Myrtaceae family are used for medicinal purpose. The oil from eucalyptus was isolated from leaves of *Eucalyptus globules*. Essential oil contains oxygenated monoterpenes namely 1, 8 –eucalyptol, α-terpineol; terpinen-4-ol.These essential oil is also used as traditional medicine as antidiabetic drug [7]..In 1980 World health organization (WHO) expert committee recommended about Diabetes mellitus that customary plant treatment for Diabetes mellitus justify further evaluation.

But it is a burning question for every drug that after getting treated, how much it is altering the blood parameters and microscopic anatomy of cell and tissue? So for that, a thorough analysis should be done to check the negative effect of drug on biochemicals and cell's microscopic anatomy.

In the present experiment, Streptozotocin induced diabetic mice were treated with eucalyptus oil at a certain dose was used to treat them without any side effects on the model.

Different biochemical parameters and cellular anatomy was thoroughly studied and can be ensure that eucalyptus oil has a tremendous effect on hyperglycemia, having a hepatoprotective as well as nephroprotective property when treated at a certain dose.

Materials and methods

Plant sample

Binomial name of the plant is *Eucalyptus globulus* Labill. These plants are commonly known as Tasmanian bluegum or southern bluegum. The plant name has been checked with http://www.theplantlist.org. The accepted name record derives from WCSP(World checklist of selected plant families) data supplied on 2012–03-23. The original publication details are Voy.Rech.Perouse 1:153 1800.

Pure extracted oil was purchased from Auroshikha,-Pondicherry and were used without further purification. To know the chemical constituents of the oil, they were chemically characterized by gas-chromatography. The condition for gas chromatography was mentioned as follows: Gas chromatography: The essential oils were analyzed using a Perkin-Elmer gas chromatograph model 8700, which is equipped with flame ionization detector (FID) and HP-5MS capillary column (30 m × 0.25 mm). Injector and detector temperatures were adjusted at 220–290 °C. Column oven temperature was set from 80 to 220 °C at the rate of 4 °C per minute. Initial and final temperatures were 3 and 10 min, respectively. Helium gas was used as carrier with flow of 1.5 mL per minute. A sample of 1.0 μL was injected, using slit mode (split ratio, 1:100). All quantification was done by a built-in data-handling program provided by the manufacturer of the gas chromatograph (Perkin-Elmer, Norwalk, CT, USA). The composition was reported as a relative percentage of the total peak area [8].

Experimental animals

Adult albino mice of average weight between 29 and 30 g of both sexes were obtained and were acclimatized in the laboratory for 2 weeks under proper aeration and at a temperature of 22°celsius and normal atmospheric pressure and also under proper supervision. They had access to food and pure water ad libitum and the stress level was 0 at the time of experiment.

Induction

Diabetes was induced by single intraperitoneal injection of Streptozotocin (STZ) (Sigma Aldrich 75%, USA) (60 mg/kg body weight in 0.1 mol/L citrate buffer, pH 4.5) into 16–18 h fasted mice. 5% glucose solution was supplied to STZ-induced mice for the next 24 h to avoid hypoglycemic mortality. After 96 h of STZ injection, blood sample (10 μL) from mice which were induced with Streptozotocin was collected by tail snip method and glucose was monitored by Accu-Chek (Roche Accu-Chek Advantage® whole-blood glucose monitor).

Fasted mice with glucose concentration greater than 200 mg/dl (11.1 mmol/L) were considered as hyperglycemic [9].Therefore initial checking of all biochemical parameters without induction of hyperglycemia was consider as day 0.After 96 h (4^{th}day) only blood glucose was checked to confirm the induction of diabetes. Then at 7th, 14th, 21th, 28th day whole biochemical parameters were observed.

Mice grouping and treatment

There were total 56 mice which were grouped into 7 sets containing 8 mice in each group.

Group I-Control (Normal diet). Group II to VII (STZ) and were diabetic. Group III (Glibenclamide [5 mg/kg] [10]. Group IV to VII (0.5%,1%,1.5%,2% Eucalyptus oil [EO]) respectively. EO was prepared by taking different percentage (0.5,1, 1.5, 2) of oil with respect to water as solvent and after immediate sonication which was done by ultrasonic sonicator stabilized the sample and immediately they were injected to the body .

Lethal dose toxicity test

EO were administered to mice at a dose of 0.5, 1, 1.5, 2, 2.5 (ml/kg body weight) and observe the behavioral change and mortality rate for a whole day.

Experiment

A 28 days treatment was done and body weight and biochemical assays were analyzed. At the end of the treatment, mice from each group's blood samples were collected from the retro orbital plexus then were sacrificed by anaesthetized. Blood serum was collected by repeated centrifugation at 3000×g for 10 min to assay blood parameters (Bilirubin, Cholesterol, SGPT, SGOT, ALP, GGT, Urea, Creatinine) For hepatoprotective enzymes like SGPT and SGOT tests modified UV (IFCC) kinetic assay was followed where absorbance was recorded at340nm.ALP was checked by p NPP –AMP (IFCC), kinetic assay. Absorbance was taken at 405 nm.GGT test was also done following the SZASZ method. ENZOPAK GGT is formulated on Szasz method recommended by Scandinavian Society for Clinical Chemistry and Physiology.This method is very particular in kinetic, specific and sensitive. Serum absorbance for GGT was taken at 405 nm.Bilirubin and cholesterol test followed Jendrassik and Grof method and CHOD-PAP method. Optical absorbance for Bilirubin was at 546 nm and 505 nm for cholesterol respectively. For nephroprotective enzymes like urea and creatinine Urease, Berthelot, end point assay and modified Jaffe's reaction initial rate assay was followed. And optical absorption was 578 nm for urea and 505 nm for creatinine. Liver, kidney and pancreas were excised. For histology, excised liver, kidney and pancreas were washed in phosphate buffer and then transfer to 10% formalin fixative solution for 48 h. All three types of tissues were processed for paraffin embedding and were sectioned of 5 μm in microtome. After staining with haematoxylin and eosin, they were examined under microscope (Axio Scope A1-Zesis) at 100X magnification.

Statistical analysis

Data expressed as Mean ± standard deviation and all analysis was carried out by Microsoft office excel and Origin software (version Pro 8.5; Origin Lab Corporation, Northampton, MA 01060, USA.

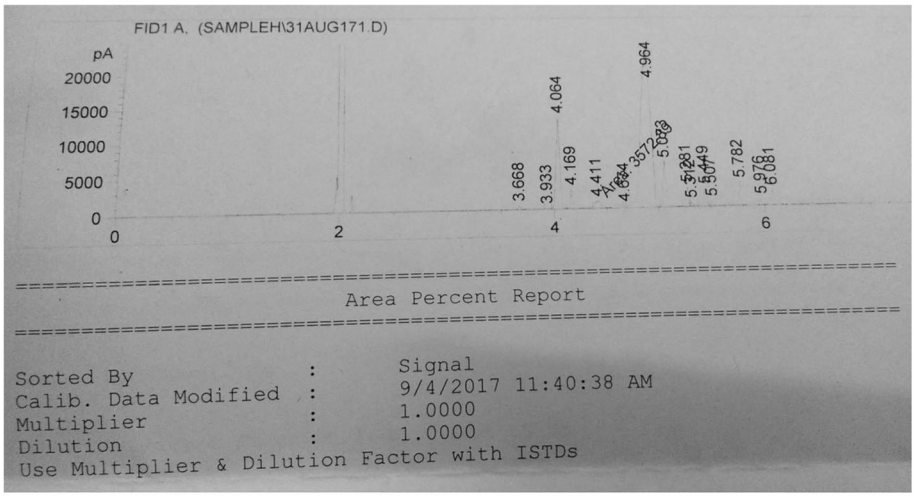
Results and discussion

Gas Chromatography results depicted in Fig. 1 showed an area percentage of 66.7% for a compound which was maximum in the chart and it has 5 min retention time so from different references of gas chromatography of *Eucalyptus globulus* essential oil which has already been established confirmed the present of 1,8-eucalyptol [11]. and Linalool (0.29%), α-eudesmol (0.34%), α- terpineol (2.66%), Terpinen-4-ol (0.33%) were also present.

Biochemical analysis

Biochemical aspects of the blood of different group of mice serum depicted through different graphs.

In Fig. 2 a] study of body weight was done, group I showed no change in body weight but due to induction of Streptozotocin only (Group II diabetic) the body weight was decreased. But positive result was observed when mice were treated with 1.5% EO (Group VI). Conventional drug Glibenclamide was also help to maintain the normal body weight as expected. [12, 13]. The reason behind the loss of body weight after treated with STZ was STZ reduces the number and size of adipocytes. Also cells were unable to oxidize glucose and incorporate into lipid. Because of that, loss of adipose mass occurred and loss of body weight was seen 1.5% EO regain the normal weight by regaining the number of adipocyets [14] Figure 2 b,] blood glucose level of healthy mice with diabetic mice and diabetes recovered mice was observed. Normal blood glucose level was less than 200 mg/dL observed in group I but Streptozotocin damage the beta cell in pancrease through necrosis which further unable to produce insulin that leads to rise in glucose level in blood. [15]. EO of different doses especially 1.5% and Glibenclamide helped to normalize the whole system. [16, 17]. Glibenclamide class sulfonyl urea helps to increase the amount of insulin production. The mechanism of action of most of the common antidiabetic drug was seen that they bind to the K_{ATP} inhibitory regulator subunit named sulfonyl urea receptor 1 in



Signal 1: FID1 A,

Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Area %
1	3.668	BV	0.0219	748.13684	0.33140
2	3.933	VV	0.0456	276.19620	0.12235
3	4.064	VV S	0.0324	3.13789e4	13.89973
4	4.169	VV S	0.0228	7082.36523	3.13724
5	4.411	MM	0.0640	3572.88672	1.58266
6	4.674	VV T	0.0160	11.31750	0.00501
7	4.964	VV S	0.0758	1.50752e5	66.77779
8	5.073	VB S	0.0267	1.20828e4	5.35226
9	5.281	VV X	0.0192	3606.42627	1.59752
10	5.312	VV X	0.0224	675.58673	0.29926
11	5.449	VV X	0.0237	4018.08398	1.77987
12	5.507	VV X	0.0172	770.18933	0.34117
13	5.782	VV X	0.0260	6006.45313	2.66065
14	5.976	VV X	0.0204	1202.01611	0.53245
15	6.081	VV X	0.0250	3568.36182	1.58066

Fig. 1 Gas Chromatography of Eucalyptus Essential Oil. A Dose Dependant hepatoprotective and nephroprotective activity of eucalyptus oil on Streptozotocin induced diabetic mice model

pancreatic beta cell. Inhibition cause cell membrane depolarization and result in opening voltage gated calcium channel and increase the amount of intracellular calcium (ca2+) in beta cell which cause stimulation of insulin release. Post effect of EO treatment has been shown a dramatic reduction of high glucose which confirms the high level of insulin which further promotes an idea that this insulin are secreted only because of the stimulation of pancreatic beta cells. So probability of mechanism of EO as drug may follow the same pathway like other conventional drug which requires further extended analysis.

In Fig. 2 c] Normal Bilirubin range was observed in control group but higher in STZ induced group. Bilirubin is the main bile pigment which formed because of breakdown of heme of red blood cells by reticuloendothelial system. Unconjugated bilirubin is carried by Albumin to the liver where it is conjugated with glucuronic acid and becomes water soluble. Total Bilirubin concentration always increases severely in hepatocellular disease. Here also increased Bilirubin was observed as STZ caused hepatocellular damage but after EO treatment again normal range of Bilirubin was seen, especially when treated with 1.5%.EO. [18, 19, 20]. Figure 2 d , showed the distinct variation of total cholesterol among normal, diabetes and diabetes recovered mice. Control group have normal range of cholesterol, followed by increased in cholesterol range in group II. Cholesterol is a waxy substance made by the body and found in some animal-based foods. Blood cholesterol levels describe a group of fats also known as

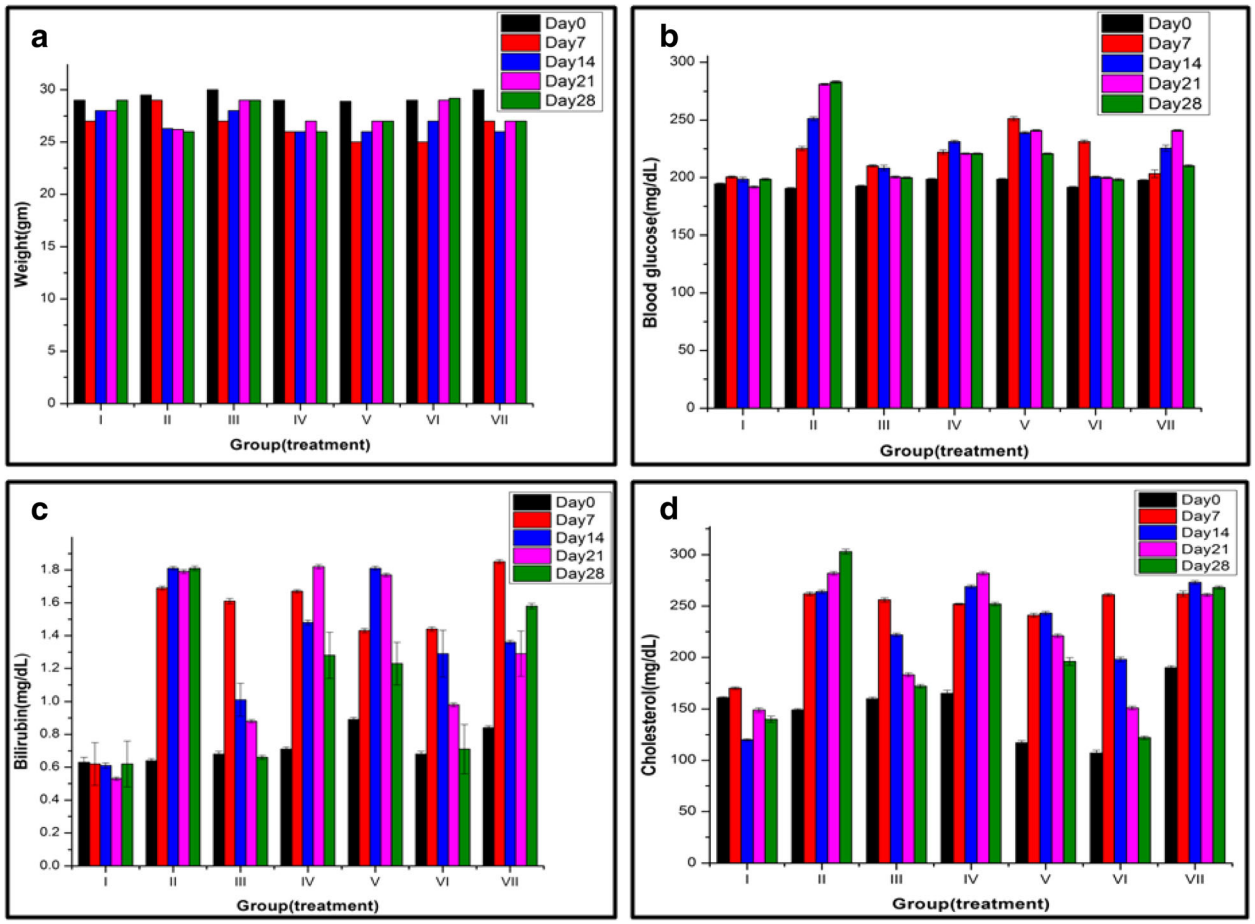


Fig. 2 **a**] Body weight, **b**] Blood glucose, **c**] Bilirubin, **d**] Cholesterol

lipoproteins that include HDL and LDL. Cholesterol can be harmful by contributing to narrowed or blocked arteries. Result showed that conventional drug Glibenclamide drags the level of cholesterol into normal range. Like the same EO of 1.5% also depicted the same result. As 261 mg/d L is quite high at 7th day but it reduces drastically to 122 mg/d L on 28th day [21].

SGPT and SGOT are elevated in viral and other liver diseases associated with Hepatic necrosis. SGPT is more liver specific enzyme. Medium increase of these enzymes may be seen in Cirrhosis, Extrahepatic cholestasis, and carcinoma of liver. When the liver cells get damaged or injured, these enzymes came into the blood stream, raising their percentage in blood levels. Hence raised blood levels of SGOT and SGPT signifies liver disease or injury. As result observed in Fig. 3 a and b where STZ induced blood serum had high SGPT & SGOT level confirming liver damage which can be rectified by EO 1.5% treatment [18, 19, 22]. Figure 3 c depicted the comparative analysis of serum level of Alkaline phosphatase (ALP) of different treated group of mice. Clinical significance of ALP showed detecting liver related diseases or bone disorders. When liver was affected by STZ, cells released increase amount of ALP into the blood stream. This test is also used to detect blocked bile ducts because ALP is high especially in the edge of cells that join to form bile duct. Elevated ALP result could confused whether they got elevated because of live or bone disorder. So for further confirmation GGT may also be done because GGT level always increased in liver diseases but not in bone disorders. Elevated ALP like 113 IU/DL had been observed in serum of STZ induced mice, which can be reduced and came to normal level when EO of different dose was used as drug especially when 1.5% EO was applied. ALP is usually much less elevated than SGPT and SGOT when bile ducts are blocked (usually gallstone, scars from surgery or by cancer. [18, 19, 21, 23]. GGT functions in the body as a transport molecule. It helps to move other molecules around the body which has a significant role in helping the liver to metabolize drugs and other toxins.GGT is concentrated in the liver, but it's also present in the gallbladder, spleen, pancreas, and

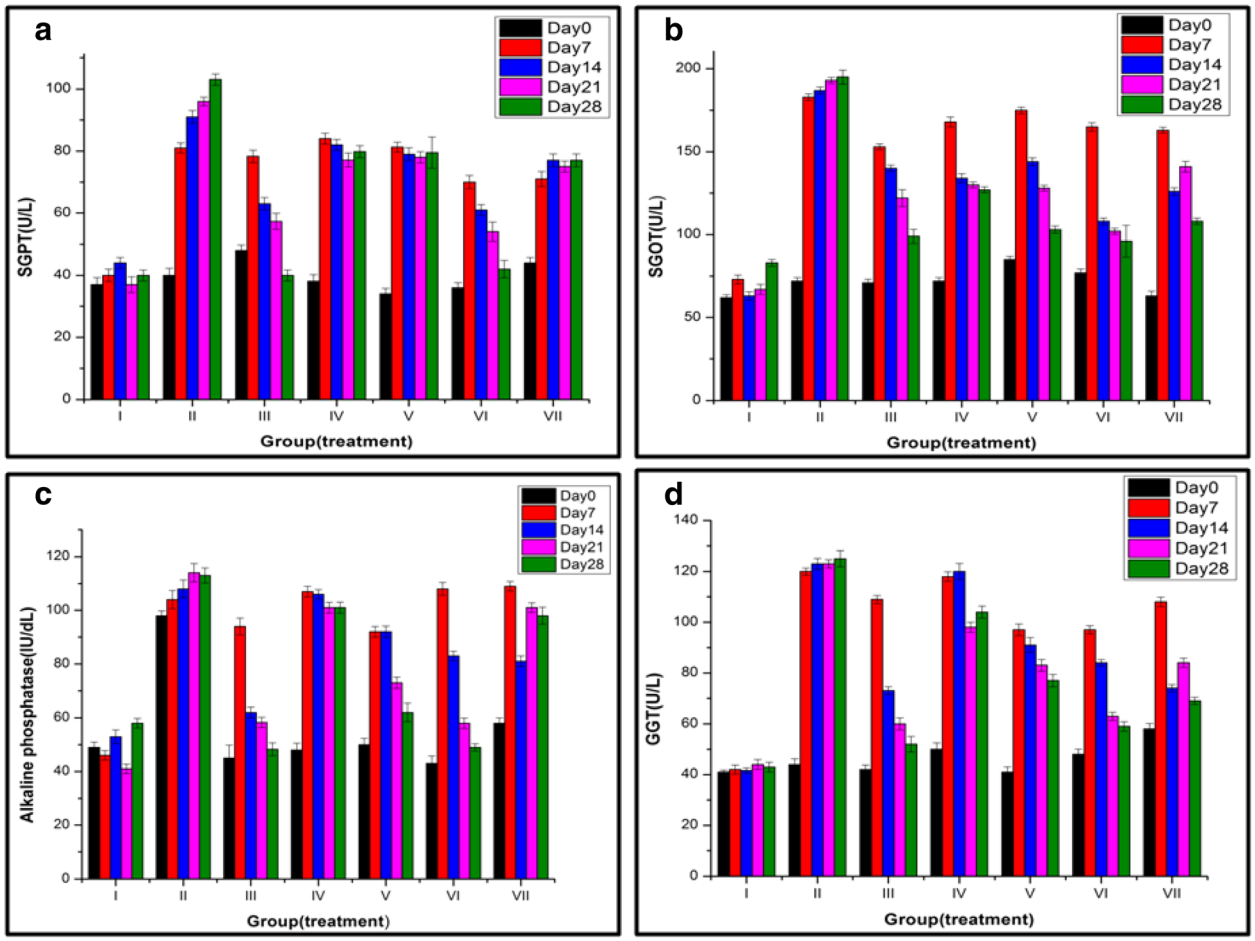


Fig. 3 [**a**] SGPT, [**b**] SGOT [**c**] ALP, [**d**] GGT

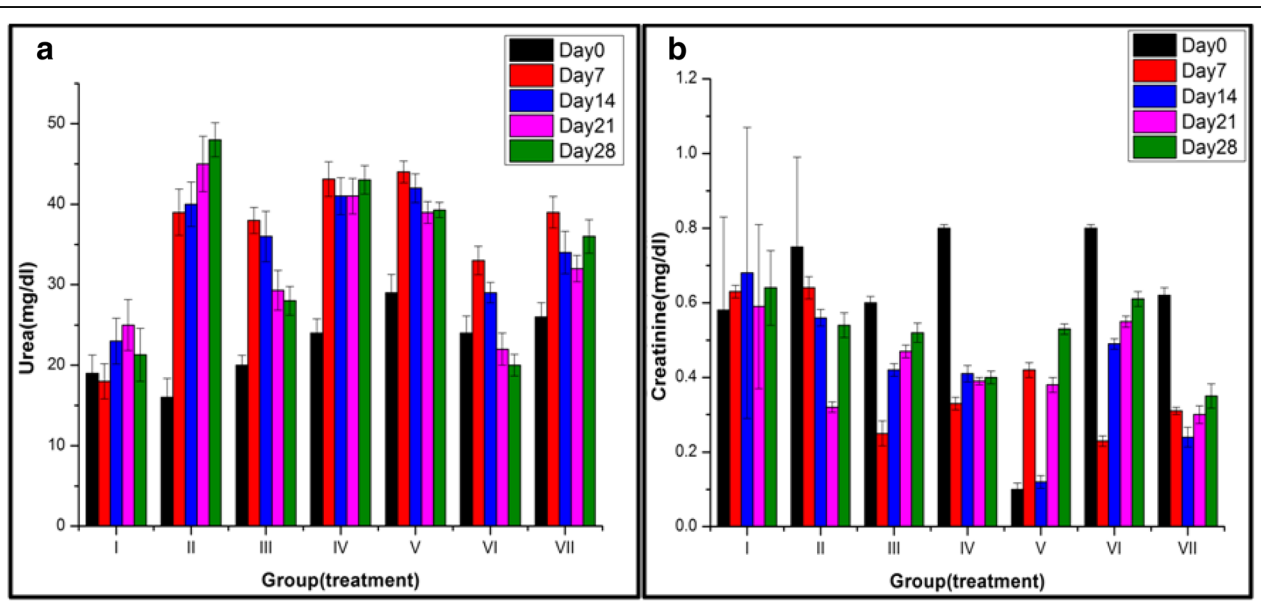


Fig. 4 [**a**] Urea, [**b**] Creatinine

kidneys. GGT blood levels are usually high when the liver is damaged. This test is often done with other tests that measure liver enzymes if there's a possibility of liver damage [24]. In this Fig. 3 d, group II has possibilities of liver damage as confirm by elevated GGT result whereas significant results could be seen in group VI where treated drug EO helped in lowering GGT to normal level [25].

Figures 1, 4 a and b are the data depicted urea and creatinine level of serum of different groups of treated mice. Both elevated urea and creatinine of the serum treated with STZ can be observed in both the figures and normalized range of these kidney enzymes were acquired by treating the model with 1.5% EO. Patient with chronic liver disease have a significantly lower baseline serum creatinine concentration than the general population [26, 27]. Urea and creatinine are the two enzymes generally used in testing nephroactivity as they are the chief indicator of kidney function. Abnormally high levels of creatinine give a warning of possible malfunction or failure of the kidney. Urea (BUN) is another indicator of kidney function.BUN to creatinine ratio generally provides more precise information about kidney function.

Lethal dose toxicity test

Survival rate was 100% and no reports of death was seen even after 28 days.

Histopathological analysis

The above figure showed the histopathological study of liver tissue which depicted the change of cell morphology before and after the treatment of eucalyptus oil(EO). In Fig. 5 a we can see the normal structure of central vein (marked) also the hepatocyte and sinusoids are well preserved and demarcated.. Cytoplasm is not vacuolated. Coming to Fig. 5 b cell morphology was not normal and having abnormal structure of central vein, vacuolated cytoplasm was observed, many hepatocytic cells were found to be necrotized near central vein. Figure 5 c tissues were treated with Glibenclamide and regeneration of

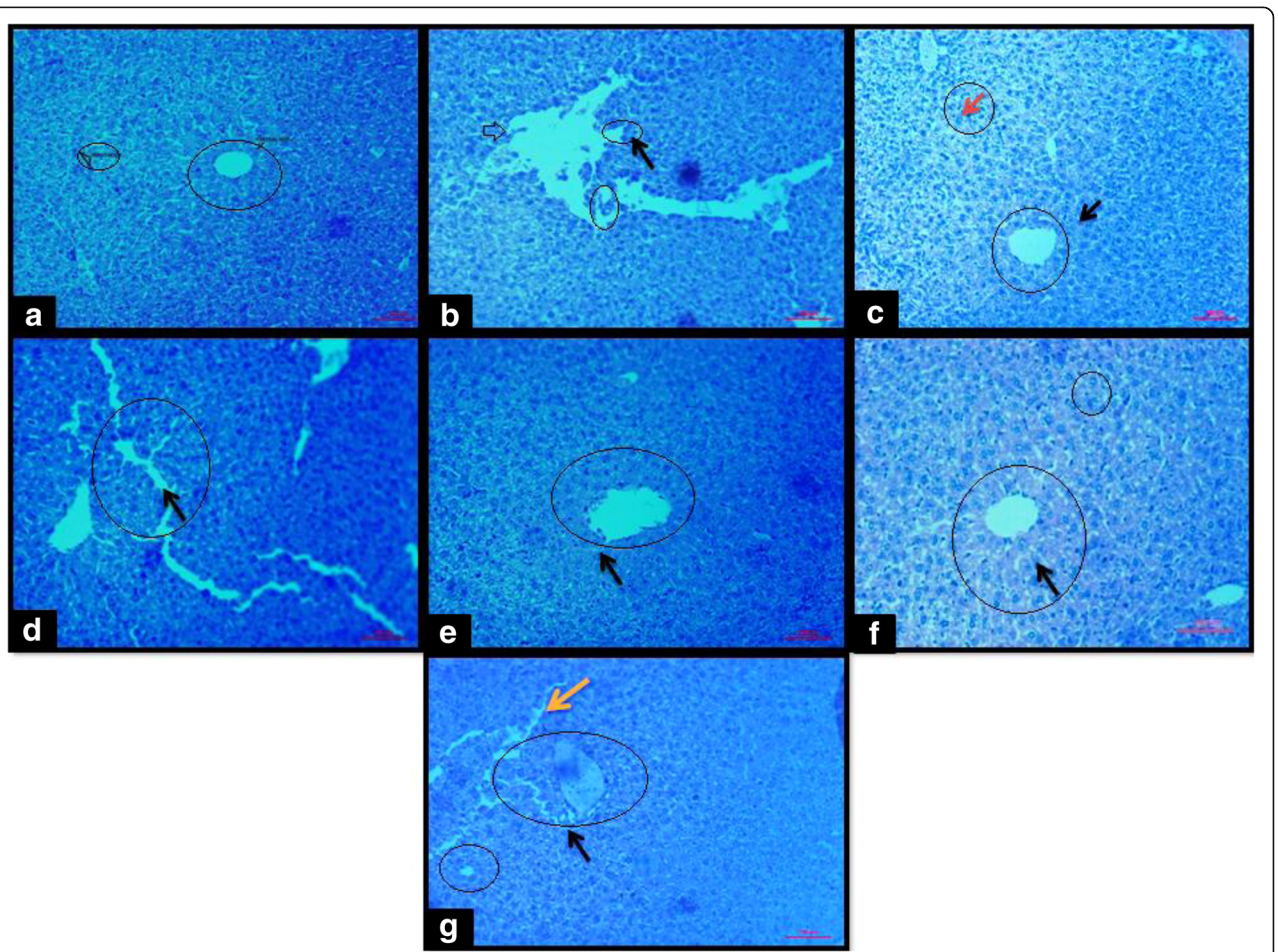


Fig. 5 Histological alteration in the liver (H&E staining 100×) photomicrograph of liver tissue, **a**. Control **b**. Only (STZ). **c** .STZ and Glibenclamide. **d**. STZ and 0.5%Eucalyptus oil (EO) **e**. STZ & 1% (EO), **f** STZ & 1.5% (EO), **g**. STZ & 2% (EO). Scale bar of 100 μm

hepatocytic cells were noticed followed by regaining the normal shape of central vein and the shape of the normal cell was regained. Figure 5 [d, e, f, g] depicted the effect of EO. And the best result showed by Fig. 5 f where 1.5%EO was applied where we can see complete structure of central vein and hepatocyte with normally visible sinusoid and less vacuolated cytoplasm was observed. Necrosis, congestion of the blood vessel and inflammatory infiltration was less observed. Overall analysis exhibited that 1.5% EO helps to restore the normal architect of liver tissue. Prominent central vein structure and hepatocytes were marked in the Fig. 5 (d, e, f, and g) [4, 28, 29, 30].

Figure 6 a showed that is control without diabetes showed the normal architect glomerulus surrounded by Bowman's capsule. Distal convoluted tubules and proximal tubules were observed. In Fig. 6 b the tissues were treated only with STZ and the structural abnormality can easily be identified having abnormal glomerulus with infiltration in proximal tubule. In Fig. 6 c the kidney tissues were healed with the help of Glibenclamide. In case of Fig. 6 d where tissues were treated with 0.5% EO, in some place shrinkage of glomeruli and tubular infiltration was noticed. But as the dose of the drug EO changed from 0.5 to 1%(Fig. 6 e) pathological percentage of damage in structure of kidney section reduced significantly and the best result was depicted by the tissue when treated with 1.5% EO (Fig. 6 f) where no messengial expansion or messengial cell proliferation or thickening of glomerulus basement membrane was observed. Regeneration of new healthy tissue was shown with normal architect. But there is no significant improvement showed by tissue treated with 2% EO (Fig. 6 g) [4, 31].

Here in Fig. 7 a showed normal tissue architecture where islet of langerhans was prominently visible with acinar cells were arranged in lobules (marked with arrows).

But in case of STZ treated tissues (Fig. 7 b), the structural morphology was not normal; size of the islet cell was shrunken with architectural disarray (marked) and number of acinar cells decrease around the islet cell. When the tissue was treated with Glibenclamide then in usual manner the tissue got recovered and normal morphology was gained gradually. When EO was treated, 0.5% didn't show

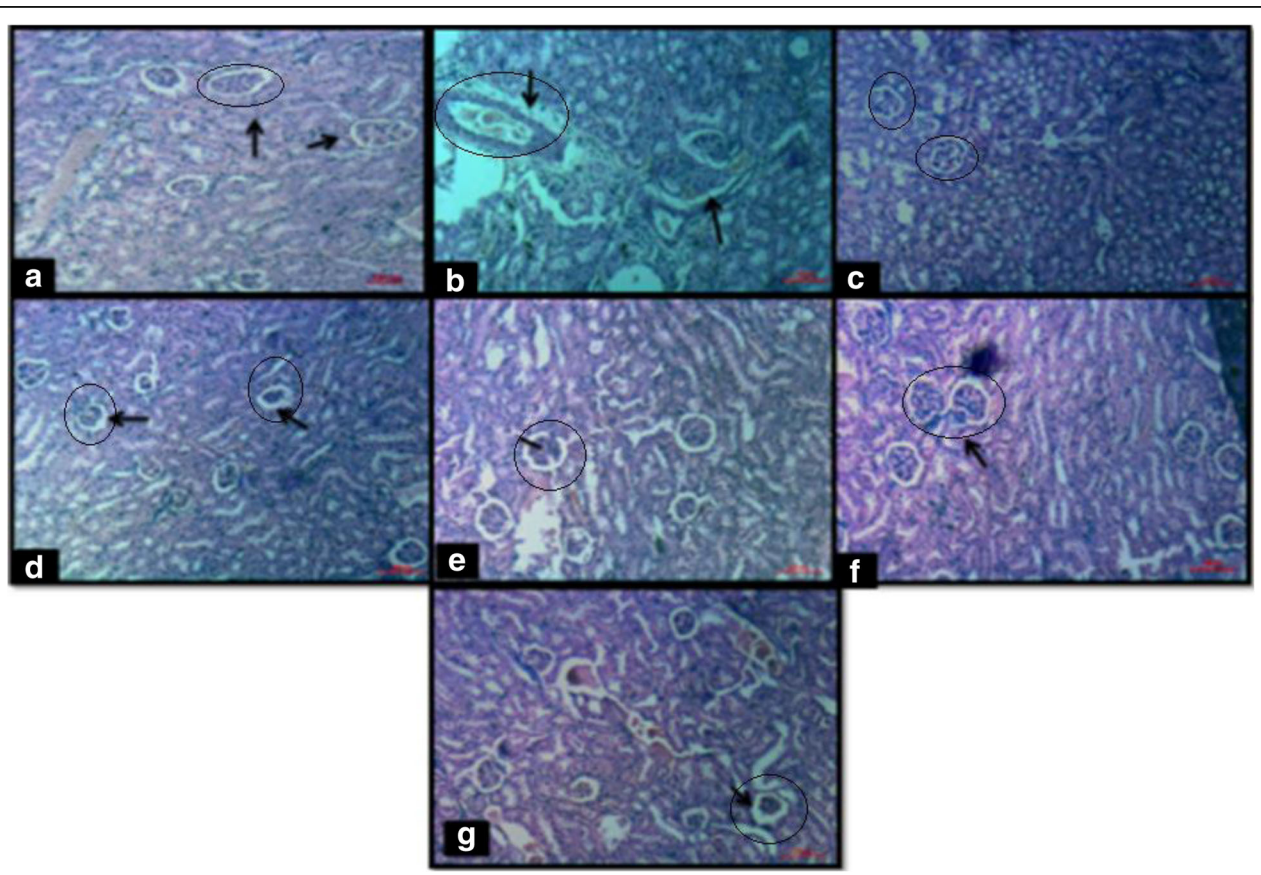


Fig. 6 Histological alteration in the kidney (H&E staining 100×) photomicrograph of kidney tissue.**a**. Control **b**. Only(STZ). **c**. STZ and Glibenclamide. **d**. STZ and 0.5%Eucalyptus oil (EO) **e**. STZ & 1% (EO), **f**.STZ & 1.5% (EO), **g**. STZ & 2% (EO). Scale bar of 100 μm

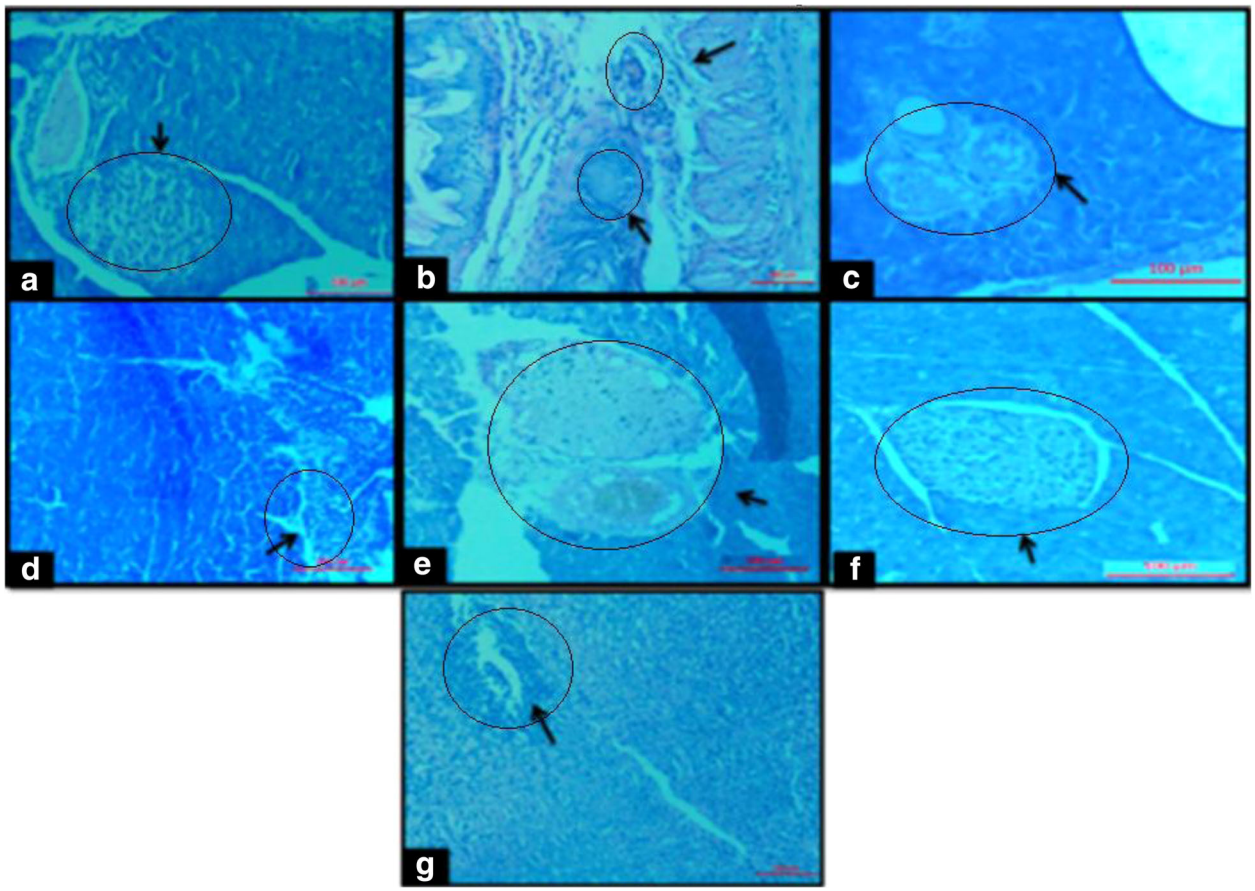


Fig. 7 Histological alteration in the pancreas (H&E staining 100×) photomicrograph of pancreas tissue. **a**. Control **b**. Only (STZ). **c**. STZ and Glibenclamide. **d**. STZ and 0.5%Eucalyptus oil (EO) **e**. STZ & 1% (EO), **f** STZ & 1.5% (EO), **g**. STZ & 2% (EO).Scale bar of 100 μm

any significant change but 1% showed proper structure of islet cells (Fig. 7 e) with increased number of acinar cells. But best result was obtained when 1.5% was treated, at that time better restoration of the beta cell in comparison to other dose was observed. Normal size of islet cells with normal number of acinar cells with less necrotic cell and contracted blood vessels were observed (Fig. 7 f). When 2% was applied there was improvement in the number of acinar cells but no improvement was took place for islet of langerhans cells [4, 32]. As the dose of Streptozotocin was 60 mg/kg body weight which has been consider as a moderate dose for the induction of diabetes and it didn't damaged all of the pancreatic cells which was proved by the Histopathological image of pancrease as shown in Fig. 7 and also proved that 1.5% dose of Eucalyptus oil has the capability to regenerate the damaged pancreatic cells. As this work is a basic preliminary work which emphasized only the dose dependant activity of the eucalyptus oil on diabetes, so insulin release was not considered as a checking parameter.

Conclusion

So the main purpose of the work is to search for an alternative substituent of antidiabetic drug which can work as potentially as conventional drug for diabetes but without having side effects which was evidenced by biochemical and histopathological analysis and also therapeutically competent at a particular dose of 1.5%.So eucalyptus oil proved to be safe and steady source for hyperglycemic patients at a particular dose. Though it is a dose dependant study of the essential oil but further research need to upgrade the information and also the molecular pathway behind the dose dependant activity need to be searched further.

Acknowledgements

The team would like to acknowledge the department of pharmacy Jadavpur University for their kind help and also acknowledge Dr. Avery Sengupta and Prof.Achntya Saha from University of Calcutta for providing us the instrument facility.

Funding

This work has not been funded by any source/organization.

Authors' contributions

Monalisa Chakraborty: Design of experiment and perform the experiment, Data analysis and interpretation, Writing section.
Biswajoy Bagchi: Design of experiment, Data analysis and interpretation, Sukhen Das, Ruma Basu, Papiya Nandy: Checking writing section, Interpretation of experimental data, significance of result.

Competing interest

The author declares that they have no competing interest.

Ethics approval

Regarding the animal ethics, consent was approved by department of pharmaceutical technology Jadavpur University, Kolkata, India.
Serial/Registration number: 1526–1805/GO/Re/S/15/CPCSEA 10/06/2015,Jadavpur University,188,Raja S.C. Mullick Road,Kolkata – 700,032,West Bengal.
Also followed the NIH guidelines for the welfare of laboratory animals and also obeys the guideline for the care and use of laboratory animals. [Eighth Edition Committee for the Update of the Guide for the Care and Use of Laboratory Animals Institute for Laboratory Animal Research Division on Earth and Life Studies (National research council.)]

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Received: 6 April 2017 Accepted: 3 December 2017
/ Published online: 27 March 2018

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