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ISOLATION AND CHARACTERIZATION OF ACTIVE COMPOUND OCTACOSANOL FROM *THUJA ORIENTALIS* CONE AND ITS ANTI-HYPERGLYCEMIC EFFECT ON STREPTOZOTOCIN INDUCED DIABETIC RAT.

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Abstract
Here, we describe the anti-diabetic properties of Octacosanol, a natural substance made from the methanolic extract of *Thuja orientalis* cone, and how it affects diabetic rats that have been induced with STZ. On the basis of spectrophotometric, mass spectrometry, NMR, and infrared data, compounds were characterized. When given orally, octacosanol reduces plasma blood glucose levels, increases body weight, and restores the histological architecture of the pancreas and liver in STZ-induced diabetic rats. It also has a positive effect on the lipid profile, liver, and kidney functions. The entire outcome was contrasted with the conventional anti-diabetic medication metformin.
Key words: Octacosanol, Steptozotocin, anti-diabetic, metformin.

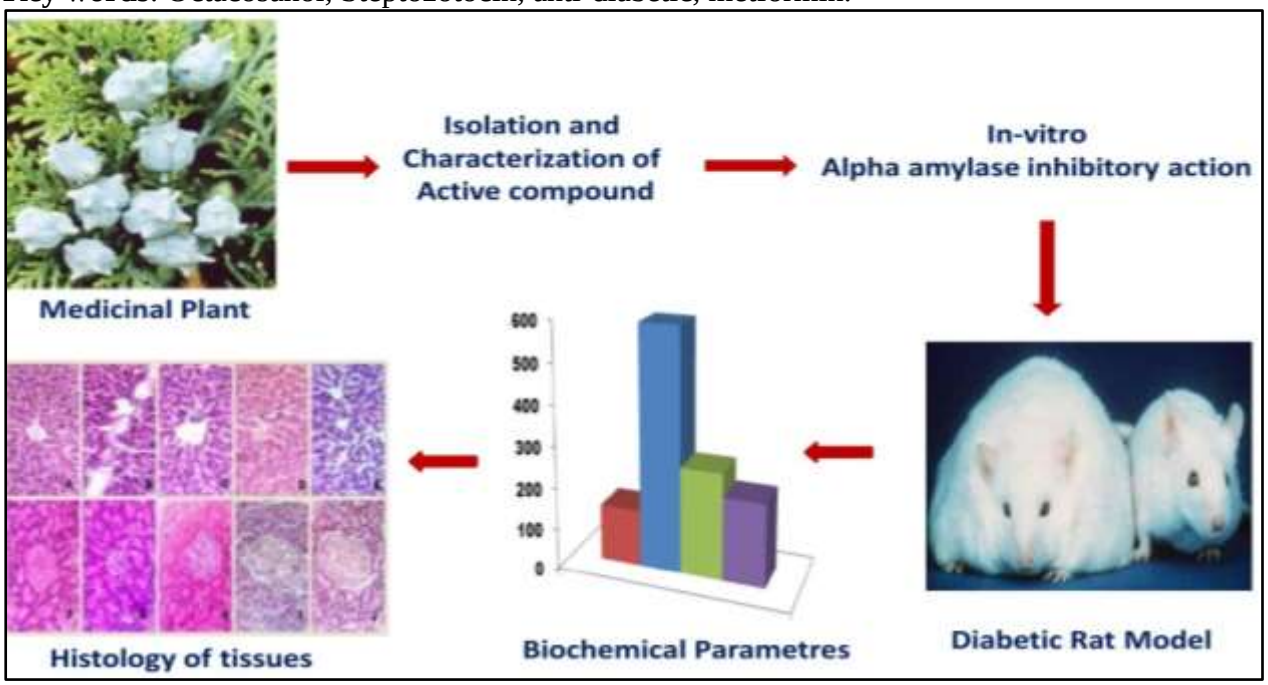

Fig.1. The natural substance Octacosanol's anti-diabetic benefits illustrated graphically.

Introduction
Natural products having high chemical diversity and biochemical specificity representing the best enduring approaches to drug discovery. Plant natural products have been the basis of traditional medicine system and World Health Organization (WHO) estimated that about 80-85 % of population relies on traditional medicine for their primary health care (Chaachouay et al. 2019). Traditional medicine and ethno botanical information have played an important role in scientific research (Wright, 2019). The underlying reason for natural products as sources of such a large proportion of existing drugs might be the similar interaction of natural products with biosynthetic enzymes and therapeutic targets (Nair and Jez, 2020).

Diabetes mellitus (DM) is a global health problem affecting 25 million numbers of people in India. There appears to be a host of potential genetic, environmental and immunological risk factors may be involved in the etiology and pathogenesis of these aberrations. DM is threatening because of the

development of many severe secondary complications, including atherosclerosis, renal dysfunction and failure, cardiac abnormalities, diabetic retinopathy and ocular disease (Shah et al. 2021). Diabetes is still not completely curable by the present anti diabetic agents. Insulin therapy is the only satisfactory approach in diabetic mellitus, even though it has several drawbacks like insulin resistance (Chen et al. 2022), anorexia, brain atrophy and fatty liver in chronic treatment (Tsimihodimos et al. 2018). Traditional medicines (TM) are a fruitful source of future drugs to counteract insulin resistance, consistent with a resurgence of interest in drug discovery from natural products. A major advantage of TM is that they have been used to treat human diseases for many years and so there is considerable knowledge concerning *in vivo* efficacy and safety, two of the confounding problems facing other new chemical entities (Tan et al. 2008).

Thuja orientalis (synonym *Platycladus orientalis*) is evergreen coniferous tree belonging to the family cupressaceae. It is a traditional medicinal plant used by ethnic people of Eastern Himalayan region. It has shown antiseptic, antimicrobial, and antioxidant as well as it is effective against warts, bronchitis, skin infection, osteoarthritis and it is good immunostimulant and diuretic (Jasuja et al. 2013). Less information is available on its antidiabetic activity. Our study is based on active compound isolation and characterization from *Thuja orientalis* cone and here we found one active compound 'Octacosanol' having antidiabetic property. We also evaluate its healthy effect on lipid profile, liver and kidney function.

2. Experimental

2.1. Collection of Plant material and chemicals

Thuja orientalis cones were collected from University of North Bengal campus, Darjeeling district (GIS location: 26°42′36″ N 88°21′06″ E) and a specimen copy submitted in the Plant Taxonomy Laboratory, Department of Botany, University of North Bengal (Accession number: NBU-11836). All the chemicals of analytical grade were purchased from Sigma-Aldrich India Limited, Hi Media, India and E. Merck, India.

2.2. Preparation of plant extract

After collection of the cones, these are dried properly in shade area and then transform into fine powder. Methanolic extract of the cone was prepared by using soxhlet extraction method. 30gm of sample was packed and extracted with 250 ml of methanol for 8 hours. In a rotary evaporator under reduced pressure the extract was concentrated at 40°C and the sample was stored at -20°C for future use (Okwori et al. 2008).

2.3. Isolation and characterization of compound

The biologically active compound was isolated from methanolic extract of *Thuja orientalis* cone. Compound was first isolated by repeated column chromatography (Tomer et al. 2009) on silica gel (pore size 60 Å). Silica in the column was dissolved by different ratio of ethyl acetate and petroleum ether. Here the active compound was isolated using 3% solvent ratio in 10 ml of each small fractions and each fraction was checked through thin layer chromatography (TLC) to find out single compound. The prepared sample has been characterized using the following experimental techniques: The FT-IR spectra were recorded on JASCO 5300 FT-IR spectrophotometer; Ultraviolet spectra were recorded on JASCO V-530 spectrophotometer; ^{1}H NMR spectra was measured in $CDCl_3$ with a **FT-NMR SPECTROMETER**, Model (BRUKER AVANCE II 400); LC-ESI-MS analysis was obtained by AGILENT 6520 Q-TOF Mass Spectrometer with AGILENT 1200 HPLC system.

2.4. *In vivo* Antidiabetic activity of active compound

2.4.1. Animals

For *in vivo* experiment, Swiss albino rats (4 weeks of age and 160-180 g BW) were purchased from authorized suppliers from Kolkata, WB. Before experiment the rats were acclimatized at 22°- 25°C and 12 hours day night cycle for 7 days (Ayele et al. 2021). Then the rats were kept in polypropylene cages and fed with standard food mixture: Wheat flour (*Triticum aestivum*): 250 g/kg, Bengal gram (*Cicer arietinum*): 150 g/kg, Maize dust (*Zea mays*):150g/kg, Milk powder (spray dried):150g/kg, Macromineral mix*: 49.75g/kg, Micromineral mix**: 0.25g/kg,

*Macromineral mix (g/kg food): $CaCO_3$: 16.7; $NaHPO_4$: 13.7; NaCl: 6; KCl: 12; MgO: 1.75.

**Micromineral mix (mg/kg food): $FeSO_4 7H_2O$: 216; $CuSO_4$: 20; $Ca(IO_3)_2$: 1.3; MnO: 5; $CoCl_2\ 6H_2O$: 1.9; Na_2SeO_3: 0.30.

All the animals were cared for in compliance with guidelines of the Institutional Animal Ethics Committee (IAEC), Department of Zoology, University of North Bengal **(The IAEC approval No: IAEC/NBU/2022/38).**

2.4.2. Acute toxicity study

The compound was studied for acute toxicity before the experiment (OECD iLibrary, 2002). The compound was consumed by the rats at a level of 2000 mg/kg BW. The animals were then observed for 4 hours to look for any signs of toxicity, including respiratory discomfort, motor activity, trembling, muscular spasm, drowsiness, diarrhoea, lack of the righting reflex, and salivation. For 24 to 72 hours, none of the animals' appearance or conduct changed noticeably, and no signs of mortality were discovered. Thus Octacosanol has been demonstrated to be safe and harmless up to a dose range of 2000 mg/kg body weight) (Pottathil et al. 2020).

2.4.3. Induction of experimental diabetes in test animals

Before induction of diabetes the rats were kept in starved and dehydric condition for 14 hours (Zhang et al. 2019). STZ was freshly prepared in cold citrate buffer (0.01M, pH 4.5) and intraperitoneally introduced at the dose of 60 mg/kg body weight. The development of diabetes was established 72 hours after STZ injection by measuring blood glucose levels from the tail tip using a glucometer strip (ACCU-CHEK, Guide). Diabetes rats were chosen based on their blood glucose levels above 300 mg/dl. From day 3 to 14, they received treatment with either octacosanol or metformin. As a vehicle, 0.01 M cold citrate buffer (pH 4.5) was administered into normal control rats.

2.4.4. Treatment of diabetic animals

The *in vivo* test was conducted for 15 days with 30 rats were distributed into five groups (n=6):

Group-I (NC): Normal control received vehicle daily.

Group-II (DC): Streptozotocin induced diabetic control received vehicle daily.

Group-III (DC+ Met): Diabetic rats treated with metformin orally (standard drug) (10 mg/kg body weight) once daily from day 3 to day 14.

Group-IV (DC+ Octacosanol 250): Diabetic rats treated with Octacosanol orally (250 mg/kg body weight) once daily by feeding in vegetable oil from day 3 to day 14.

Group-V (DC+ Octacosanol 500): Diabetic rats treated with Octacosanol orally (500 mg/kg body weight) once daily by feeding in vegetable oil from day 3 to day 14.

2.4.5. Analytical procedure

2.4.5.1. Measurement of body weight

On day 0, 3, 5, 10 and 15 of the treatment, total body weight of all experimental rats were recorded.

2.4.5.2. Estimation of blood sugar level

On day 0, 3, 5, 10 and 15 of the treatment, blood sugar level was determined by glucometer strip (ACCU-CHEK, Guide) from the tail tip.

2.4.5.3. Collection of serum

On day 15, blood was drawn from a rat's tail vein for serum collection (Parasuraman et al. 2010). Blood was taken and stored at room temperature for 30 minutes in an uncontaminated centrifuge tube and centrifuged at 20°C and 1500 g for 10 minutes. Without disrupting the pellet, the serum supernatant was removed and kept at 4–8°C for various biochemical tests.

2.4.5.4. Study of serum biochemical parameters

2.4.5.5. Lipid profile analysis

2.4.5.5.1. Estimation of total cholesterol

Estimation of total cholesterol in serum sample was analysed by commercially available kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) (Robinet et al. 2010). In this assay cholesterol esterase is used to hydrolyze cholesteryl ester into free cholesterol. Then cholesterol oxidase acts on free cholesterol to produce a chemical which reacts with a probe to generate colour and it is measured at 500 nm.

2.4.5.5.2. Estimation of triglycerides

Estimation of triglycerides in serum sample was analysed by commercially available kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) (Saravanam et al. 2017).

In this assay, triglycerides are converted to free fatty acids and glycerol. Then glycerol is oxidized to generate a product which reacts with a probe to generate colour which is measured at 500 nm.

2.4.5.5.3. Estimation of HDL

Estimation of HDL in serum sample was analysed by commercially available kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) (Marz et al. 2017). Here serum sample is reacted with polyethylene glycol by which all LDL and VLDL are precipitated. HDL then remains in the supernatant then assayed as a sample for cholesterol using CHOD/PAP reagent.

2.4.5.6. Liver function test (SGOT, SGPT)

SGOT and SGPT were measured by IFCC method (International Federation of Clinical Chemistry) described in the commercially available kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) (Rasyid et al. 2020). Here SGOT (EC 2.6.1.1) catalyses the transfer of amino group from aspartate to α-ketogluterate forming oxaloacetate and glutamate. Glutamate is detected in a reaction that reacts with probe to generate colour which is measured at 340 nm. Here SGPT (EC 2.6.1.2) catalyses the transfer of amino group from alanine to α-ketogluterate forming pyruvate and glutamate. Pyruvate is detected in a reaction that reacts with probe to generate colour which is measured at 340 nm.

2.4.5.7. Kidney function test (Urea, creatinine)

2.4.5.7.1. Estimation of Urea

Urea in the serum was estimated by Urenase/GLDH method as described in the commercially available kit (Agappe Diagnostics LTD, Kerala, India) (Langenfeid et al. 2021). In this assay, urea reacts with compounds in the presence of enzymes to form a product that reacts with OxiRed probe to generate a colour product measured at 340 nm. OD is directly related with the concentration of urea.

2.4.5.7.2. Estimation of Creatinine

Creatinine in the serum was estimated by commercially available kit (Agappe Diagnostics LTD, Kerala, India) (Narimani et al. 2020). Here, creatinine reacts with picric acid under alkaline condition to produce colour product measured at 546 nm. OD is directly related with the concentration of urea.

2.4.5.8. Histology

The liver and pancreas were removed from the rats and kept in PBS (Phosphate Buffer Saline), then fixed in FAA solution. After paraffin infiltration, solid sections of tissues were cut at 5 μm thickness by microtome and stained with eosin and hematoxylin. After mounting by DPX, the sections were examined under a compound microscope at a magnification of 40X and photographed (Tamizhazhagan and Pugazhendy, 2017).

3. Results and discussion

3.1. Characterization of compound

The compound has a molecular weight of 408, is monoacetate, and a melting point of 80°C, and looks like white fibres. The UV end absorption peak was seen at 224 nm. In the IR spectrum, the hydroxyl group and double bond contain bands at 3398.04 cm^{-1} and 1630 cm^{-1}, respectively. Aliphatic C-H stretching vibrations and aliphatic methylene stretching vibrations initially occurred at 2848 cm^{-1} and 2916 cm^{-1}, respectively. Methylene produced audible scissoring and rocking vibrations at 1465 cm^{-1} and 721 cm^{-1}, respectively. The isolated molecule must consequently have a straight chain structure with more than seven carbon atoms, as inferred from the IR spectra. Six terminal methyl protons showed as a triplet at δ 1.08 ppm in the ^{1}H NMR spectrum of $CDCl_3$, and two of the terminal protons first appeared as a triplet at δ 0.88 ppm (J = 4.95 Hz). A broad singlet at δ 1.44 and δ 1.21 ppm revealed the existence of 20 methylene protons. The hydroxy proton re-vibrated at δ 3.57 ppm as a singlet. The methine proton's hydroxyl group resonated at δ 3.44 ppm. Olefinic protons resonated at δ 4.80 ppm (J = 10 Hz) and δ 4.84 ppm (J = 8 Hz). The hydroxyl group's neighbouring carbon resonated at δ 71.99 ppm, while the olefinic carbon resonated at δ 123.45 ppm. Methyl and methylene's end carbons echoed at concentrations of δ 14.03, 25.63, and 25.32 ppm, respectively. Other methylenes resonated at respective concentrations of δ 29.30 ppm and δ 29.66 ppm. A peak for the molecular ion $(M+1)^{+}$ at m/z 409 in its mass spectrum was identified as being **$C_{28}H_{56}O$**. The substance was given the name Octacosane-10-ol-21-ene or Octacosanol as a result and considered as active compound (**Figure 1**).

3.2. Effect of Octacosanol on plasma glucose and body weight

When compared to the normal rats (NC), the body weight (BW) of the diabetic control rats (DC) was found to be significantly reduced at the end of the 15th day. Oral administration of either Octacosanol (250 and 500 mg/kg BW) or metformin (10 mg/kg BW) to diabetic rats increased the body weights significantly when compared with DC. Octacosanol at the doses (250 and 500 mg/kg BW) increased the body weight to 186.50 ± 17.56 (+ 5.86%) and 187.67 ± 19.06 (+ 12.59%) respectively when compared to DC (-20.63% lose BW) on 15th day (**Figure 2b**).

The antidiabetic effect of Octacosanol was evaluated by measuring the fasting blood glucose levels on day 0, 3, 5, 10 and 15. The fasting blood glucose level of DC increased significantly ($p<0.001$) when compared with NC after 72 h of STZ insertion. Before the administration of metformin and Octacosanol, the fasting blood sugar levels of the four DCs (Diabetic Control, Positive Control, Treated Group I, Treated Group II) were almost similar on day 3. The fasting blood glucose level reduced to its lowest point on 15th day of treatment with 46.23 and 53.51% reduction at 250 mg/kg and 500 mg/kg of Octacosanol, respectively. When compared to DC, metformin (10 mg/kg) resulted in 65.02% drop in fasting blood glucose level at the end of the study (**Figure 2a**).

3.3. Effect of Octacosanol on histology of liver and pancreas

STZ induced gr. II rat's liver revealed significant destruction of hepatocytes. Some portion showed total absent of hepatocytes indicates internal haemorrhage. Drug induced gr. IV and V rat's liver showed well improved the structure of liver, increase cell number and regained their shape. Similar type of improvement found in gr. III rat treated with metformin.STZ induced gr. II rat's pancreas revealed significant destruction and reduction in size of Islets. Drug induced gr. IV and V rat's pancreas overcome the damage and regained their structure and normal size. This result compared with metformin (gr. III) showed similar type of improvement of histological architecture (**Figure 3**).

3.4. Lipid profile analysis, liver & kidney function test

The lipid profiles of normal and STZ-induced diabetic rats were examined by monitoring total cholesterol, triglycerides and HDL-cholesterol levels. In Diabetic Control (DC), cholesterol and triglyceride level increased up to 164 and 194 mg/dl respectively while HDL level reduced up to 32 mg/dl. Rats treated with Octacosanol (gr. IV and V), cholesterol and triglyceride level reduced up to 94, 74 and 101, 97 mg/dl respectively while increase HDL level up to 49 and 56 mg/dl. Metformin decreased cholesterol and triglyceride level up to 78 and 98 mg/dl respectively while increase HDL level up to 54 mg/dl (**Figure 4**).

In DC, SGOT and SGPT level increased up to 77 and 109 mg/dl respectively. Rat treated with Octacosanol (gr. IV and V), SGOT and SGPT level decreased up to 58, 51 and 79, 72 mg/dl respectively. Metformin decreased SGOT and SGPT level up to 47 and 67 mg/dl respectively (**Figure 5**).

In DC, urea and creatinine level increased up to 65 and 1.46 mg/dl respectively. Rat treated with the compound (gr. IV and V), urea and creatinine level decreased up to 37, 31 and 0.71, 0.62 mg/dl respectively. Metformin decreased urea and creatinine level up to 33 and 0.56 mg/dl respectively (**Figure 5 and 6**).

Conclusion

In conclusion, the compound Octacosanol derived from the methanolic extract of *Thuja orientalis* cone, exhibit anti-hyperglycemic activity in STZ-induced diabetic rat. It reduces the blood sugar level, increase body weight and restores the damaged histological architecture of pancreas and liver. Additionally it showed healthy impact on plasma lipid profile as well as liver and kidney function. Further studies are needed to establish the detailed mechanism of action of octacosanol in blood glucose homeostasis.

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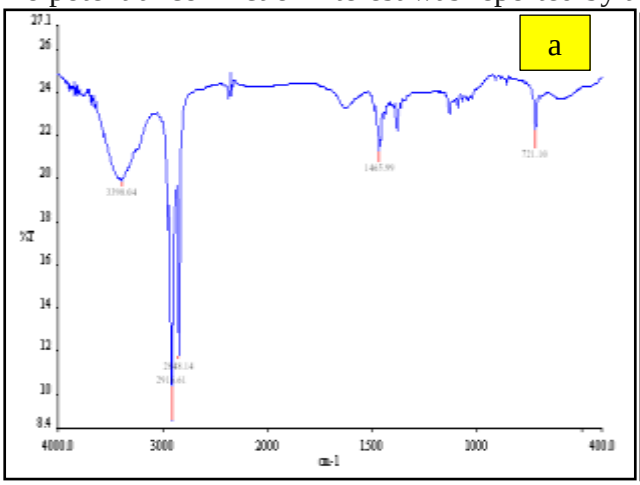
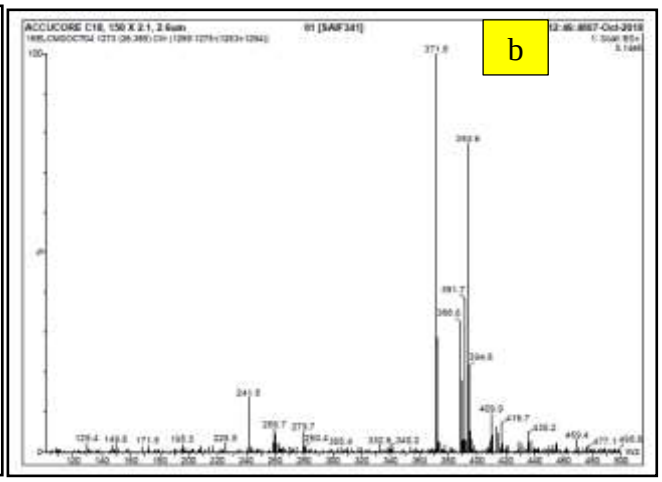
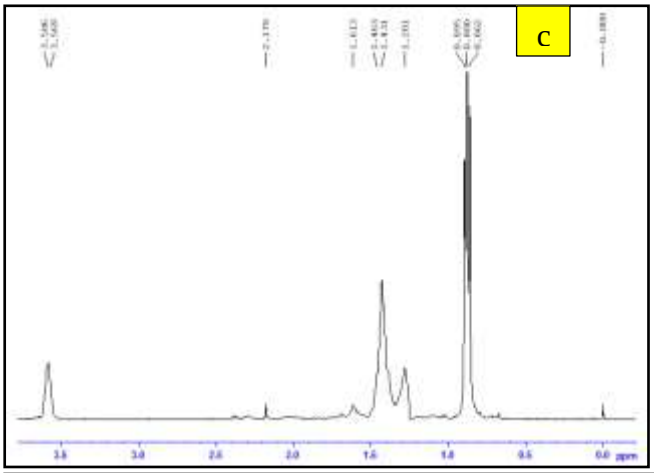
Disclosure statement

No potential conflict of interest was reported by the authors







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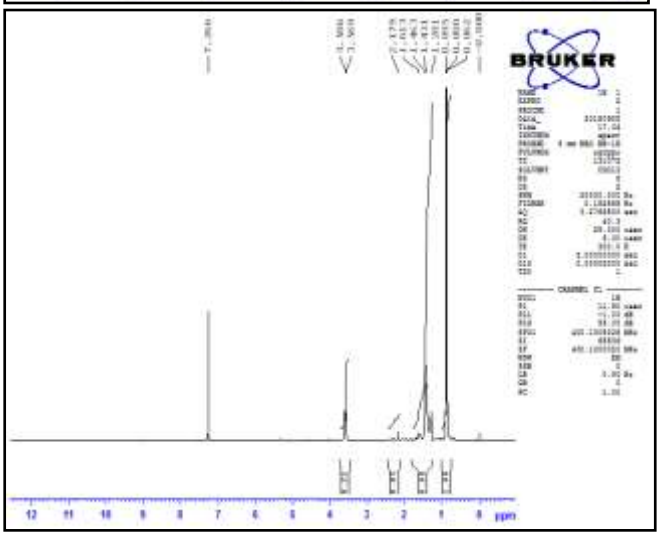

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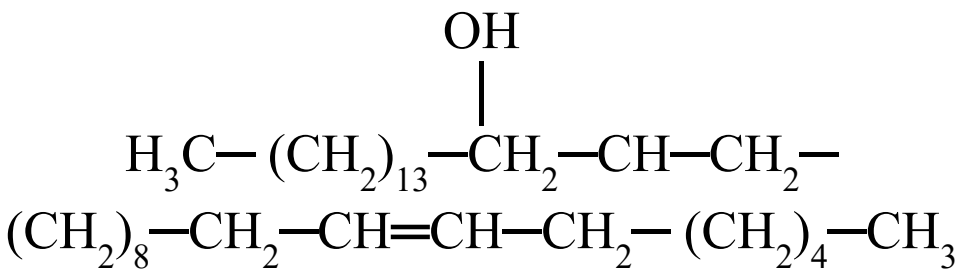

Figure 1: Structural analysis of the compound derived from methanolic extract of *Thuja orientalis* cone. (a) IR data, (b) Mass spectrometry data, (c) and (d) NMR data, (e) Chemical structure of the compound.

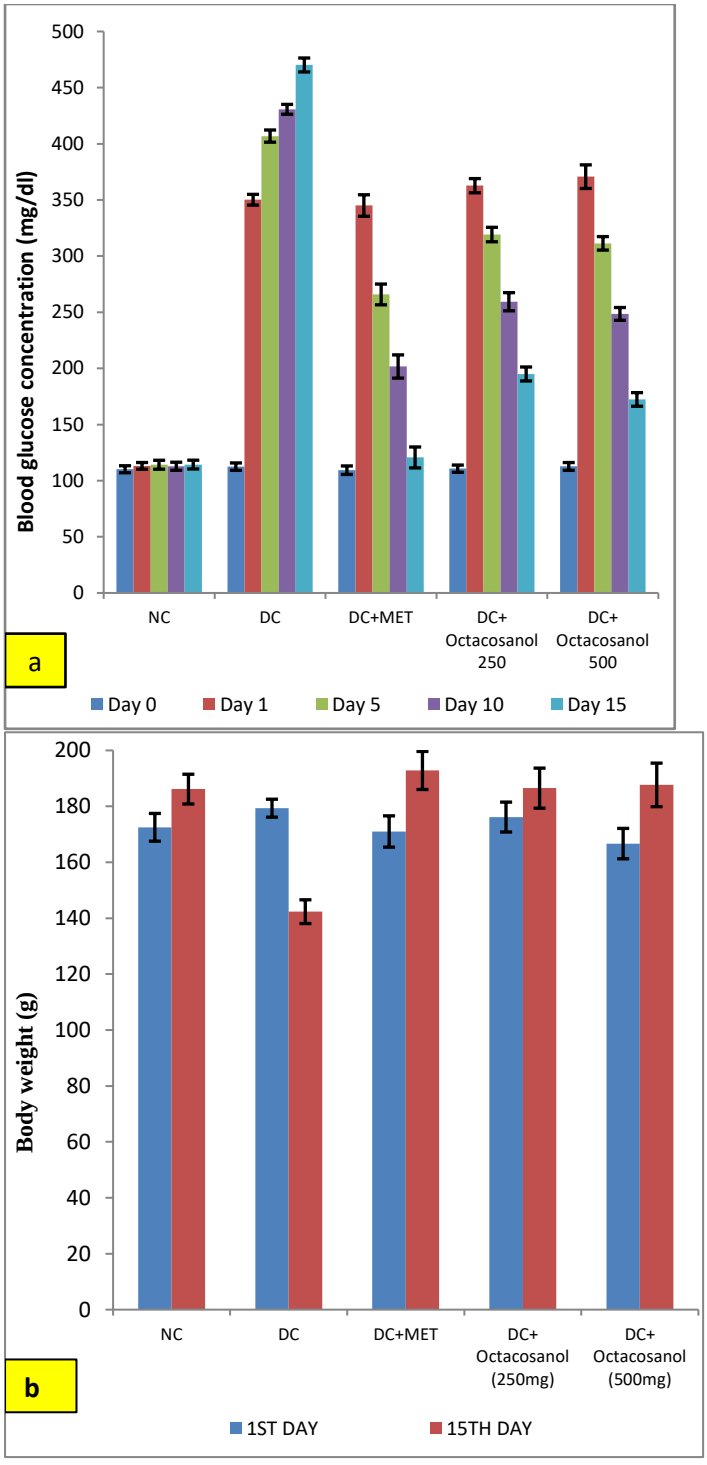

Figure 2: Effect of 15 days treatment of octacosanol on plasma glucose (mg/dl) (a) and body weight (g) of STZ induced diabetic rat (b). Results are compared with metformin. $P< 0.05$ compared with normal and diabetic control.

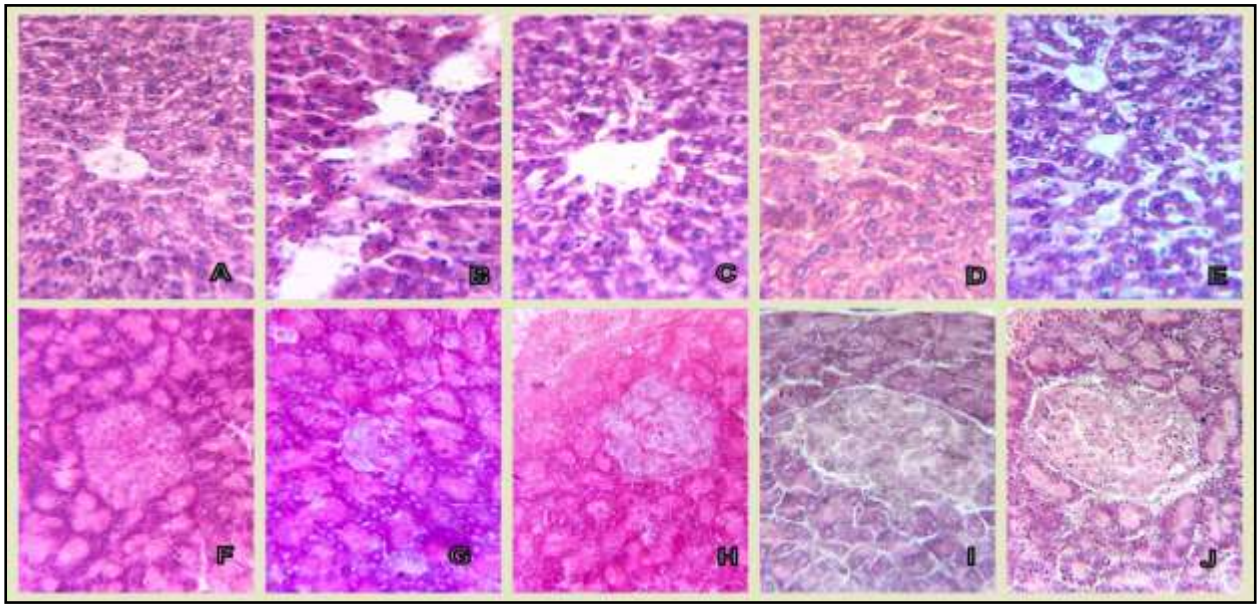

Figure 3: Effect of 15 days Octacosanol treatment on histology of liver and pancreas. Liver: (A) Normal Control, (B) Diabetic Control, (C) Positive Control, (D) Treated Group I, (E) Treated Group II; Pancreas: (F) Normal Control, (G) Diabetic Control, (H) Positive Control, (I) Treated Group I, (J) Treated Group II. All the figures observed under 400X magnification (Objective 40X; Eye piece 10X)

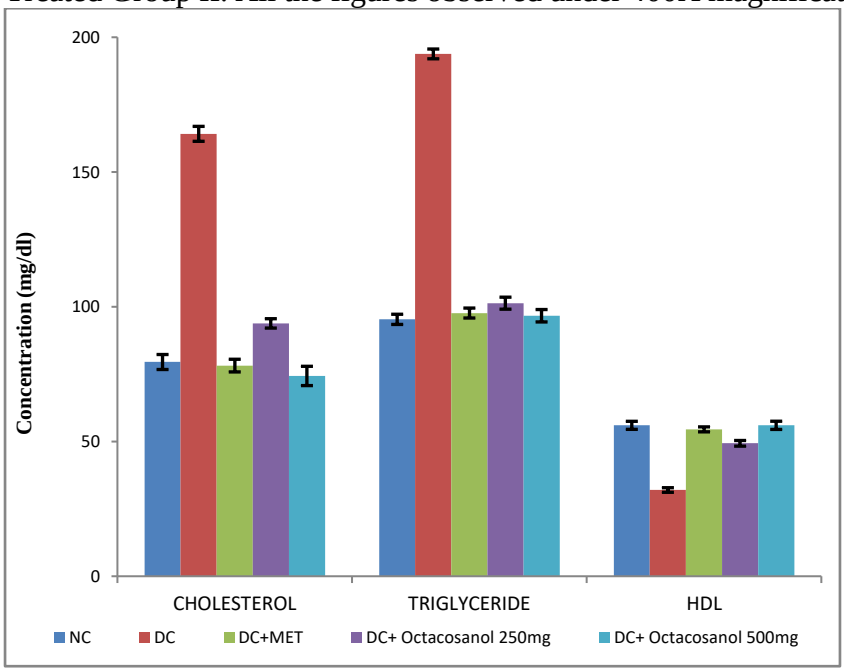

Figure 4: Effect of 15 days treatment of octacosanol on plasma cholesterol, triglyceride and HDL activities on STZ induced diabetic rat. Results are compared with metformin. $P< 0.05$ compared with normal and diabetic control.

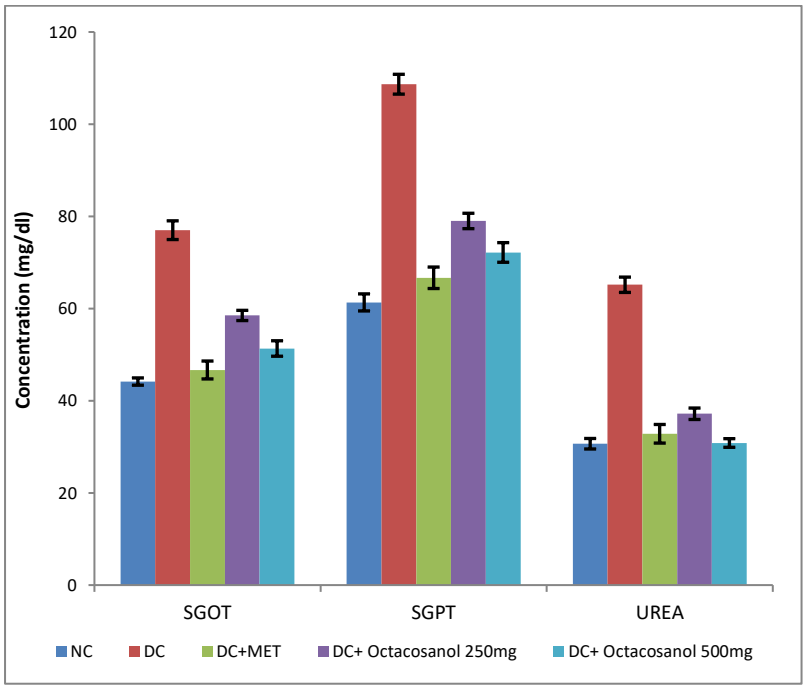

Figure 5: Effect of 15 days treatment of octacosanol on plasma SGOT, SGPT and urea activities on STZ induced diabetic rat. Results are compared with metformin. *P*< 0.05 compared with normal and diabetic control.

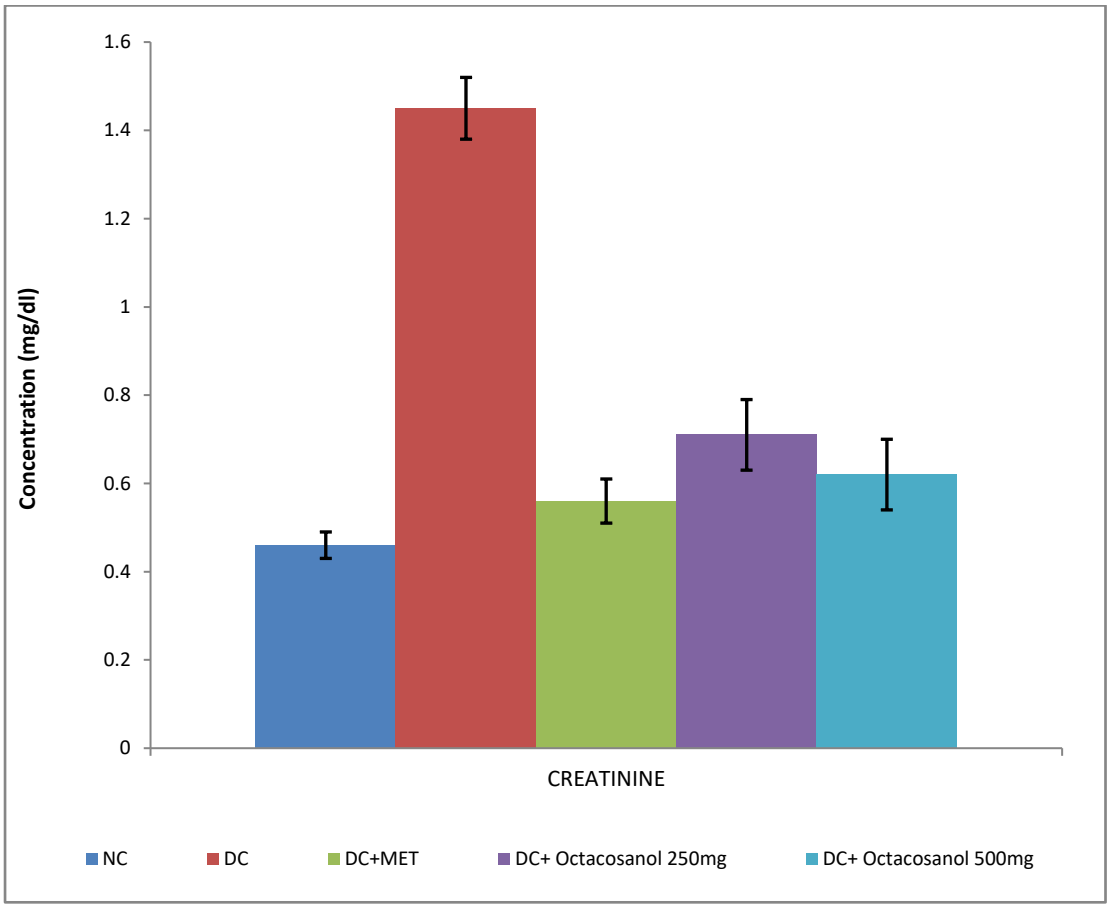

Figure 6: Effect of 15 days treatment of octacosanol on plasma creatinine activities on STZ induced diabetic rat. Results are compared with metformin. *P*< 0.05 compared with normal and diabetic control.

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