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Protective role of *Excoecaria agallocha* L. against streptozotocin induced diabetic complications

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Abstract
Oxidative stress has a pivotal role in the pathogenesis and development of various diabetic-related complications, the most common and debilitating effects of diabetes mellitus are on kidney, liver and heart tissue. There is accumulating evidence that *Excoecaria agallocha* L extract, a rich source of phenolic and flavonoid components, has hypoglycemic and antioxidative properties. This study aimed to determine the protective effects of *Excoecaria agallocha* L extract against streptozotocin-induced diabetic complications on kidney, liver and heart in rat. The diabetic rat model was generated by intraperitoneal injection of streptozotocin (STZ) at a dose of 60 mg/kg, i.p. dissolved in 0.01M citrate buffer (pH 4.5) after 16 h fasting. A subset of the STZ-induced diabetic rats intragastrically administered with *Excoecaria agallocha* L. extract (250 and 500 mg/kg/day) before or after the onset of diabetes for 16 weeks, whereas other diabetic rats received only isotonic saline as the same volume of *Excoecaria agallocha* L extract. To evaluate the effects of *Excoecaria agallocha* L extract on the diabetic rats various parameters, including oxidative parameters, liver enzymes, kidney-specific parameters, and histopathology were assessed along with other biochemical assessments. Greater blood sugar, SGOT, SGPT, creatine kinase, urea whereas lower uric acid, creatinine and total protein were detected in the STZ-diabetic rats, which were significantly attenuated after *Excoecaria agallocha* L extract administration, dose-dependently. Even various oxidative stress parameters were also drastically changed in a STZ-diabetic rats which were improved by *Excoecaria agallocha* L extract. Histopathological study of liver, kidney and heart tissue were supported the potential protective effect of *Excoecaria agallocha* L extract. The activity elicited by the extract might be due to its ability to activate antioxidant enzymes. The findings suggest the potential use of the extract of *Excoecaria agallocha* L. as a novel therapeutically useful nephron protective, hepatoprotective and cardio protective agent.

Keywords: *Excoecaria agallocha* L, STZ, antioxidant, hepatoprotective, nephron protective, cardio protective

1. Introduction
Diabetes Mellitus is a constitutional disease. It is also known as "Disease of Civilization" (Urbanization) seen more in cities than villages. But however, it is now making inroads into Indian villages. Diabetes is an important human ailment afflicting many from various walks of life in different countries. It is an outcome of sedentary lifestyle & incorrect food habits. No. of people afflicted by Diabetes Mellitus is increasing each day. 9-12% population of world has either established Diabetes Mellitus (or) tendency of contracting it in near future [1, 2].18 Millions of Indians are suffering from Diabetes Mellitus. Though it is rampant today, it is not a new disease. It is well-known from historic times. Well known ayurvedic physicians Maharshi Charaka (600BC) & Sushrutha (400BC) correctly described almost all the symptoms of this disease and called it as "Madhu Meha" (a shower of honey) and explained in Ayurvedic literature called 'Sushrutha Samhitha' [3-6].

Nature has provided us plenty of medicinal plants and in fact, all of them have not been explored yet. The medicinal value of plant lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive components of the plants are alkaloids, flavonoids tannins, and phenolic compounds. These are reports that plant possessing phenolic compounds, flavonoids and tannins are useful as antioxidants [7, 8]. Keeping these points in view, *Excoecaria agallocha* L. is a mangrove species that belongs to Euphorbiaceae family and widely occurs on the south-east coast of India. Traditionally, *Excoecaria agallocha* L was used to treat sores and stings from marine creatures, for treating ulcers, as a purgative and an emetic, and the smoke from the bark to treat leprosy Scientifically E. agallocha was reported to have antifilarial, antioxidant, cytoprotective, antifouling, antinociceptive and piscicidal activities [9-11].

This is due to the presence of diterpenoids, triterpeneoids, flavonoid and phorbole esters [12-14]. The leaves of the plant have rich in polyphenols and flavonoids due to the presence of these constituents the plant may be has antioxidant property. Diabetic Mellitus is usually conveyed by excessive production of free radicals, hyperglycemia-induced mitochondrial reactive oxygen species production could be a key episode in the progress of diabetic complications [15-17]. The antioxidant property of the plant has deteriorates the progression of diabetic-induced complications.

Material and Methods

Identification and Authentication of Plant Materials

The plant *Excoecaria agallocha* L. identified and authenticated by Dr. Madhava Chetty, department of botany, S.V. University, Tirupati and preserved in herbarium for further identification.

Collection of Plant Materials

The leaves of *Excoecaria agallocha* L. collected in the month of April – May at coastal region of Andhra Pradesh. The collected materials are washed and dried in shade. The dried materials are powdered by using a mixer. The powdered drug is subjected to solvent extraction by soxhlet apparatus.

Extraction Procedure

Fine powder of *Excoecaria agallocha* L. is subjected to sox elation by using 70% ethanol as solvent for a period of 72 hours at the temperature of 60 °C obtain dark colored extract is cooled, filtered and concentrated by evaporation and freeze-dried. The extract thus obtained is used for further experimental studies. Dried extract is dissolved in distilled water for animal administration [18, 19].

Preliminary Phytochemical Studies [20]

Qualitative phytochemical analysis [21]

The extract obtained by solvent extraction method is subjected to qualitative phytochemical analysis in order to identify the nature of constituents present in the prepared extract solution.

The detailed study about the phytochemical test procedure as follows

A. Test for carbohydrates

Molisch's test: 2-3 ml of extract is added with 1ml of freshly prepared alpha-naphthol solution in alcohol, shaken and added conc.H2SO4 from sides of the test tube under tap water. Violet ring is formed at the junction of two liquids.

Benedict's test: To the extract solution, Benedict's reagent is added, heated on water bath and red color is formed.

Fehling's test: To the extract, equal quantities of Fehling's solution A and B is added, heated in boiling water bath for 5-10 minutes and brick red precipitate is formed.

Barfoed's test: To the 1ml of extract solution, 1ml of Barfoed's reagent is added and heated on water bath. Red precipitate is formed.

B. Test for Glycosides

Solution A

200 mg of powder is dissolved in water, filtered. To the filtrate, 5 ml of sulphuric acid solution is added, heated for few minutes. To this add 5% NaOH solution and 0.1 ml of Fehling's A and B solution is added, heated for 2 minutes, red precipitate is formed.

Solution B

200 mg of powder is dissolved in water, filtered and distilled water is added, heated for few minutes, 0.1 ml of Fehling's A and B solution is added. Heated for 2 minutes, red precipitate is formed.

Then compared the solution B with A.

Borntrager's test: A few ml of dilute sulphuric acid is added to the extract solution, boiled, filtered and treated the filtrate with chloroform and shaken well. Then separated the chloroform layer and tested with a few ml of ammonia solution. The ammoniacal layer formed pink or red color.

Modified Borntrager's test: Few ml of 5% Fecl3 solution and dilute hydrochloric acid is added to the extract. Then boiled for 5 min, cooled and shaken well with organic solvent. Then added equal quantity of dilute ammonia. The ammoniacal layer formed pinkish red color.

Legal's test: Extract is dissolved with pyridine and made alkaline with sodium nitroprusside solution. The solution becomes pink or red.

Ballet's test: 1 ml of sodium picrate solution is added to the extract. Color changes from yellow to orange.

Keller-killani test: Powdered drug extract is added to the 10ml of 70% ethanol for 2 min, then filtered and added 10 ml of water, 0.5 ml of strong solution of lead acetate and filtered. The filtrates are shaken with 5 ml of chloroform and separate the chloroform layer and evaporated. To this 3 ml of glacial acetic acid is added and cooled. Then added 2 drops of 5% ferric chloride solution. Transferred the contents into a test tube containing 2 ml of concentrated sulphuric acid. Reddish brown layer was formed at the junction of the two liquids and the upper layer slowly becomes bluish green on standing.

C. Test for Flavonoids

Shinoda test: To the ethanolic extract of powder, magnesium turnings or foil and conc. Hcl are added. Intense cherry red color or orange-red color is formed.

General tests: To the small quantity of residue, lead acetate solution is added. Yellow color precipitate is formed. Addition of increasing amount of sodium hydroxide to the residue shows yellow coloration, which decolorizes after addition of acid.

D. Test for Saponins

Foam test: Small quantity of ethanolic extract and 20 ml of distilled water is added, shaken in a graduated cylinder for 15 minutes, 1 cm layer of foam is developed.

Hemolytic test: Drug extract is added to one drop of blood, Placed on the glass side. Hemolytic zone appears.

E. Test for Steroids

Libermann-Burchard test: Extract is dissolved in few ml of chloroform, 3 ml of acetic anhydride and conc.H2SO4 is added from sides of the test tube, bluish green color is formed.

Salkowski test: Extract is dissolved in few ml of chloroform and added equal volume of conc.H2SO4 blue or red color is formed.

F. Test for Alkaloids
Hager's test: 1 ml of extract is added to 3 ml of Hager's reagent (saturated aqueous solution of picric acid). A yellow colored precipitate indicates the presence of alkaloids.
Wager's test: 1 ml of the extract is added to 2 ml of Wager's reagent (iodine in potassium iodide) formation of reddish brown precipitate indicates the presence of alkaloids.

G. Test for Gums
Mucilage test: To the 10 ml of aqueous extract, 25 ml of absolute alcohol is added slowly with constant stirring. Filtered the precipitate and dried in air. The precipitate is examined for its swelling properties and for the presence of carbohydrates.

Hydrolytic test: The test solution is hydrolyzed using dilute HCl. Benedict's and Fehling's tests are performed. Red color is formed.

H. Test for Fixed Oils and Fats
Spot test: Press a small quantity of extract between the filter paper. Oil stains on the paper indicates fixed oils.

Saponification test: To the 1 ml of the extract, few drops of 0.5N alcoholic KOH is added and a drop of phenolphthalein is added. Heat the mixture on water bath for 1-2 hours. Formation of soap or partial neutralization of alkali occurs.

I. Test for Acidic Compounds
a) To the extract added NaHCO3 solution. Effervescence is observed.
b) To the extract added water, warmed and filtered. Litmus paper turns to blue color.

J. Test for Amino Acids
Ninhydrin test: To the small quantity of extract, 2 drops of freshly prepared 0.2% Ninhydrin reagent (0.1% solution in n-butanol) is added. Heated for few minutes, blue color is not formed.
Biuret test: 1 ml of 40%NaOH solution and 2 drops of 1% copper sulfate solution is added to the 1ml of extract. Blue color is not formed.
Xanthoproteic test: To the test solution 1 ml of conc. nitric acid is added and boil. Yellow precipitate is formed. After cooling 40% sodium hydroxide solution is added orange color is not formed.
Tryptophan test: To the 3 ml of test solution, a few drops of glyoxalic acid and conc.H2SO4 are added. Reddish violet at the junction of two layers is not formed.
Cysteine test: To the 5 ml of test solution, few drops of 40%NaOH and 10% lead acetate solution are added, boiled precipitate of lead sulfate is not formed.

K. Test for Tannins
Gelatin test: To the extract, 1% gelatin solution containing sodium chloride is added. A white precipitate is formed.

L. Test for Phenols
Ferric chloride test: To the extract, 3-4 drops of ferric chloride solution is added. Bluish-black color is formed.

Pharmacological Studies

Acute Toxicity Studies

Experimental animals

Inbred Swiss albino mice (20-35 g) of male sex are obtained from the animal house, Tirupati. The rats are maintained in a well-ventilated room with 12:12 hour light/dark cycle in polypropylene cages. Standard pellet feed (Hindustan Lever Limited., Bangalore) and drinking water is provided ad libitum throughout experimentation period. Rats are acclimatized to laboratory conditions one week prior to initiation of experiments. Ethical committee clearance is obtained from IAEC (Institutional Animal Ethics Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

Acute oral toxicity study

The procedure is followed by using OECD 423 (Acute Toxic Class Method). The acute toxic class method is a stepwise procedure with four mice of a single-sex per step. Depending on the mortality or morbidity status of the rats and the average two to three steps is necessary to allow judgment on the acute toxicity of the test substance. This procedure results in the use number of rats while allowing for acceptable data-based scientific conclusion. The method used to defined doses (2000, 1000, 500, 50, 5 mg/kg body weight) the results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of chemicals which cause acute toxicity.

Experimental procedure

Male albino mice weighing 20-35 gm are used for the study. The starting dose level of *Excoecaria agallocha* L. is 500, 1500, 2500, 5000 mg/kg body weight p.o. Dose-volume is administered to overnight fasted mice ad libidum. Food is withheld for further 3-4 hours after administration of *Excoecaria agallocha* L. and observed for signs for toxicity. The body weight of the mice before and after administration are noted that changes in skin, eyes, mucous membranes, respiratory, circulatory, autonomic, central nervous system, motor activity and behavior pattern are observed and also signs of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma are noted. The onset of toxicity and signs of toxicity also noted (OECD 2002).

Experimental Animals

Adult male or female Wister rats, weighing 150 to 200g are used in the study. The study protocol is reviewed and approved by the institutional animal ethical committee and conforms to the Indian national science academy guidelines for the use and care of experimental animals in research. Animals are obtained from the Raghavendra Enterprises, Bangalore. Rats are housed in polyacrylic cages (38X23X10 cm) with not more than four animals per cage. They are housed in an air-conditioned room and are kept in standard laboratory conditions under natural light and dark cycle (approximately 14 h light / 10 h dark) and maintained humidity 60 ± 5% and an ambient temperature of 25 ± 2°C. All experiments are performed between 9:00 am and 4:00 pm. The animals are free access to standard diet and tap water ad libitum and allowed to acclimatize for one week before the experiments. Commercial pellet diet contained 22 % Protein, 4% Fat, 4% Fiber, 36% Carbohydrates and 10% Ash w/w, supplied by Raghavendra Enterprises, Bangalore is used.

Compound (drugs to be administered) preparation

- **Extract**: Extract is weighed according to rat body weight and dissolved in distilled water.
- **Extract Dose Selection**: Based on toxicity studies

Excoecaria agallocha L. extract have selected 250 and 500mg/kg b.w dose having the good activity against streptozotocin-induced diabetic complications. Starting point of study is animal selection and randomly dividing them into 4 groups (by considering animal body weights).

- Streptozotocin Injection 60mg/kg body weight [22]

Induction of Experimental Diabetes: [23]

Study Groups

Following the induction of diabetes, the animals were randomly allocated into four groups (n =10) and treated for 16 weeks as follows:

- GROUP-1: Control (non diabetic) rats.
- GROUP-2: Diabetic rats with no treatment.
- GROUP-3: Diabetic rats treated with the *Excoecaria agallocha* L. extract 250 mg/kg per day by gavage, started 5 days prior to STZ and continued for 16 weeks.
- GROUP-4: Diabetic rats treated with *Excoecaria agallocha* L. extract 500 mg/kg per day by gavage, started 5 days prior to STZ and continued for 16 weeks. This was a randomized study with concurrent control and appropriate blinding.

Induction of Diabetes

Diabetes was induced by single injection of streptozotocin (STZ) at a dose of 60 mg/kg, i.p. dissolved in 0.01M citrate buffer (pH 4.5) after 16 h fasting. 72h after STZ injection diabetes was confirmed in rats showing blood sugar level greater than 250 mg/dL. Animals with blood glucose levels greater than 250 mg/dL were considered for further study.

Sampling and Biochemical Analysis

Blood samples were collected 72 hours after STZ administration, and thereafter every 4 weeks from orbital plexus by pricking a needle under ketamine anesthesia. Glucose-Oxidase assay method was used to determine the blood glucose. After collecting blood samples were centrifuged for 10 minutes at 3000rpm. The obtained clear sera are stored at refrigerator for subsequent measurement of SGOT, SGPT, CK, LDH, blood urea, creatinine, uric acid and total protein levels using colorimetric assay kits, according to the manufacturer's instructions.

Preparation of Tissue Homogenates

The organs (kidney/liver/heart) are removed and dissected free from the surrounding fat and connective tissue. Each tissue is longitudinally sectioned and kept at -8°C. Subsequently homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4) followed by centrifuged at 5000rpm for 10 min at 4 °C. The resulting supernatant of each organs is used for the determination of – malondialdehyde (MDA) content (kidney sample) and antioxidant enzyme levels such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH).

Histopathological Examination

At the end of the experiment, organs are immediately fixed in 10% buffered neutral formalin solution. The tissue are carefully embedded in molten paraffin with the help of metallic blocks, covered with flexible plastic molds and kept under freezing plate to allow paraffin to solidify. Cross section (5µm thick) of the fixed renal tissues is separated. These sections are stained with hematoxylin and eosin (H&E) and visualized under light microscope to study the light microscopic architecture of the dissected organs.

Statistical Analysis

The results are expressed as mean ± standard deviation (SD) differences in groups for biochemical estimations. Statistical analysis is determined by one way – analysis of variance (ANOVA), individual groups are compared with control group using Dunnett's t-test. P value < 0.05 has been considered as statistical significance level.

Results

Phytochemical Analysis

The table 1 indicates ethanolic extract of *Excoecaria agallocha* contain chief chemical constituents like carbohydrates, glycosides, flavonoids, saponins, steroids, alkaloids, gums, fixed oil and fats, tannins and phenols.

Acute Toxicity Studies

The body weight of the mice`s before and after administration of drugs is noted and the changes in the body weight are not so prominent. No changes in skin, fur, eyes, mucous membrane, respiratory, circulatory, autonomic, central nervous system, motor activity and behavior pattern are observed and also no sign of tremors, convulsions**,** salivation, diarrhea, lethargy, sleep and coma are noted. The onset and signs of toxicity is also observed. No mortality is observed. So select the dose levels of 250mg/kg and 500 mg/kg b.w (Table 2).

Effect of *E. agallocha* leaves (EAL) extract on Bodyweight

There is slight decrease of body weights non-significantly in diabetic treated group (II) when compared with control group (I). There is slight dose-dependent increase of body weights significantly in animals treated with EAL 250 mg/ kg and 500 mg/kg (III, IV) when compared with acetaminophen group (II) (Table 3).

Effect of *E. agallocha* extract on Biochemical parameter

SGOT (Serum glutamate oxaloacetate transferase):

Serum SGOT concentrations are significantly increased in the Diabetic treated group of animals compared to the normal animals indicating the induction of severe hepatotoxicity. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) decrease in concentrations of serum SGOT compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group (Table 4). SGPT (serum Glutamate Pyruvate transaminase):

Serum SGPT concentrations are significantly increased in the Diabetic treated group of animals compared to the normal animals indicating the induction of severe hepatotoxicity. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) decrease in concentrations of serum SGPT compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group (Table 5).

CK (Creatine kinase): Serum CK concentrations are significantly increased in the Diabetic treated group of animals compared to the normal animals indicating the induction of severe damage of heart tissue. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) decrease in concentrations of serum CK compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group (Table 6).

Urea: 0 week not showing significant differences into all groups and 4 week onwards showing Serum urea concentrations are significantly increased in the Diabetic

treated group of animals compared to the normal animals indicating the induction of severe nephrotoxicity. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) decrease in concentrations of serum urea compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group (Table 7).

Uric acid:0 week not showing significant differences into all groups and 4 week onwards showing Serum uric acid concentrations are significantly decreased in the Diabetic treated group of animals compared to the normal animals indicating the induction of severe nephrotoxicity. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) increase in concentrations of serum uric acid compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group (Table 8).

Creatinine: 0 week not showing significant differences into all groups and 4 week showing Serum creatinine concentrations are significantly increased (4 week) in the Diabetic treated group of animals compared to the normal animals indicating the induction of severe nephrotoxicity. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) decrease in concentrations of serum creatinine compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group. After 4-week Creatinine shows reverse profile (Table 9).

Total protein: 0 week not showing significant differences into all groups and 4 week onwards showing Serum total protein concentrations are significantly decreased in the Diabetic treated group of animals compared to the normal animals indicating the induction of severe nephrotoxicity. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) increase in concentrations of serum total protein compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group (Table 10).

Blood sugar: 0 week not showing significant differences into all groups and 4 week onwards showing blood sugar concentrations are significantly increased in the Diabetic treated group of animals compared to the normal animals indicating the induction of severe diabetes. Treatment with the ethanolic extract of *EAL* shows significant (Group C & D) decrease in concentrations of blood sugar compared to the Diabetic treated group. EAL 500mg/kg group shows more effective when compared with 250mg/kg group (Table 11).

Anti-oxidant parameters

Kidney tissues: The activity of CAT in the diabetic treated group is significantly decreased when compared to the normal animals (Group A). Treatment with the ethanol extract of *EAL* significantly (Group C & D) prevented decrease in the level of catalase activity compared to the diabetic-induced rat (Group B). Renal SOD activity is decreased significantly in the diabetic treated (group B) animals compared to normal group. Treatment with the extract (250 &500 mg/kg body wt) (Group C &D) significantly elevated the SOD levels as compared to the diabetic-induced (Group B) animals. The GSH level reduced significantly along with increased in MDA concentration in the diabetic treated group as compared to the Group A. On treatment with ethanolic extract of EAL, the GSH level is found to be enhanced significantly and the MDA contents are reduced in Group C and D as compared to the induced group (Group B) (Table 12).

Heart tissues: The activity of CAT in the diabetic treated group is significantly decreased when compared to the normal animals (Group A). Treatment with the ethanol extract of *EAL* significantly (Group C & D) prevented decrease in the level of catalase activity compared to the diabetic-induced rat (Group B). SOD activity is decreased significantly in the diabetic treated (group B) animals compared to normal group. Treatment with the extract (250 &500 mg/kg body wt) (Group C &D) significantly elevated the SOD levels as compared to the diabetic-induced (Group B) animals. The GSH level reduced significantly along with increased in MDA concentration in the diabetic treated group as compared to the Group A. On treatment with ethanolic extract of EAL, the GSH level is found to be enhanced significantly and the MDA contents are reduced in Group C and D as compared to the induced group (Group B) (Table 13).

Liver tissues: The activity of CAT in the diabetic treated group is significantly decreased when compared to the normal animals (Group A). Treatment with the ethanol extract of *EAL* significantly (Group C & D) prevented decrease in the level of catalase activity compared to the diabetic-induced rat (Group B). SOD activity is decreased significantly in the diabetic treated (group B) animals compared to normal group. Treatment with the extract (250 &500 mg/kg body wt) (Group C &D) significantly elevated the SOD levels as compared to the diabetic-induced (Group B) animals. The GSH level reduced significantly along with increased in MDA concentration in the diabetic treated group as compared to the Group A. On treatment with ethanolic extract of EAL, the GSH level is found to be enhanced significantly and the MDA contents are reduced in Group C and D as compared to the induced group (Group B) (Table 14).

Table 1: Results of phytochemical analysis

Physico chemical Tests	Ethanolic extract of *E. agallocha*
1)Test For Carbohydrates	
a)Molisch's Test	+
b)Fehling's Test	+
c) Borfoed's Test	+
2)Test For Glycosides	
a)Solution A	+
b)Solution B	+
c)Borntrager's Test	+
d)Modified Borntrager's Test	+
e)Legal's Test	+
f)Keller Killani Test	+
3)Test For Flavonoids	
a)Shinoda Test	+
b)General Test	+
4)Test For Saponins	

a)Foam Test	+
5)Test For Steroids	
a)Liberman - Buchard Test	+
b)Salkowski Test	+
6)Test For Alkaloids	
a)Hager's Test	+
b)Wager's Test	+
7)Test For Gums	
a)Mucilage Test	+
b)Hydrolytic Test	+
8)Test For Fixed Oils And Fats	
a)Spot Test	+
b)Saponification Test	+
9)Test For Acidic Compounds	
a)Extract + NaHCO3 Solution	-
b)Extract + Water, Warm, Litmus paper turns to Blue colour	-
10)Test For Aminoacids	
a)Ninhydrin Test	-
b)Biuret Test	-
c) Xanthoproteic Test	-
d)Cysteine Test	+
11)Test For Tannins	
a)Gelatin test	+
12)Test For Phenols	
a)Extract + FeCl3	+
+: Presence of compounds -: Absence of compounds	

Table 2: Results of Acute toxicity studies

S. No	Groups	Dose (mg/kg b.w.)	Signs of Toxicity	Onset of Toxicity	Duration of Study
1	*E. agallocha*	500mg	No	Nil	14 days
2	*E. agallocha*	1500 mg	No	Nil	14 days
3	*E. agallocha*	2500mg	No	Nil	14 days
4	*E. agallocha*	5000mg	No	Nil	14 days

Table 3: Effect of *E. agallocha* leaves (EAL) extract on Body weight

Group	Treatment	Dose	Change In B.W.(G)
A	Control	Vehicle	10.20±3.80
B	Streptozotocin	Streptozotocin Injection 60mg/kg b.w. (i.p.)	-2.22±1.32
C	EAL	EAL -250mg/kg.+ Streptozotocin 60mg/kg b.w. (i.p.)	6.88±2.83
D	EAL	EAL -500mg/kg.+ 60mg/kg b.w. (i.p.)	8.19±3.59

Table 4: Effect of *E. agallocha* leaves extract on SGOT

Group/Treatment	SGOT				
	0 week	4 week	8 week	12 week	16 week
Control	65.47±3.12	65.02±2.19	64.10±1.92	63.28±0.94	63.54±2.12
Diabetic****	114.84±4.34	122.27±2.98	124.94±2.49	125.53±1.2	126.46±2.93
EAL (250mg/kg)***	75.25±2.53	76.28±1.26	75.98±3.25	79.15±2.85	74.95±1.85
EAL(500mg/kg)**	69.25±2.58	71.45±2.35	68.65±1.27	69.11±0.25	68.15±2.58

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 5: Effect of *E. agallocha* leaves extract on SGPT

Group/Treatment	SGPT				
	0 week	4 week	8 week	12 week	16 week
Control	47.51± 2.50	48.15 ±0.26	49.28± 0.99	47.21 ±1.50	49.29± 1.34
Diabetic****	105.12± 1.25	106.24± 1.45	105.78± 1.05	106.37 ±1.98	109.45± 3.52
EAL (250mg/kg)***	85.21 ±3.25	89.12± 2.54	84.21 ±1.23	86.14 ±2.58	80.25 ±2.58
EAL(500mg/kg)**	65.52 ±2.54	66.57± 2.89	64.14± 2.64	61.08± 2.49	59.12± 4.51

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 6: Effect of *E. agallocha* leaves extract on CK

Group/Treatment	CK				
	0 week	4 week	8 week	12 week	16 week
Control	85.01± 2.51	84.05± 1.25	80.15± 2.6	75.12± 1.36	79.12± 3.69
Diabetic**	150.12± 2.58	145.61± 2.58	135.21± 1.25	120.14± 2.56	115.25± 3.65
EAL (250mg/kg)**	98.21± 2.15	95.54± 1.25	94.52 ±1.25	96.12 ±2.36	99.63± 2.12
EAL(500mg/kg)*	89.12 ±2.58	85.18± 2.28	84.52± 1.35	79.12± 2.53	80.12 ±1.23

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 7: Effect of *E. agallocha* leaves extract on Urea

Group/Treatment	UREA				
	0 week	4 week	8 week	12 week	16 week
Control	48.36± 3.18	50.13± 1.25	49.82 ±2.56	50.36 ±2.58	47.15 ±1.02
Diabetic*	50.13 ±2.35	95.12 ±1.29	98.15± 1.87	99.17 ±2.56	99.25 ±1.87
EAL (250mg/kg)*	48.99± 2.38	66.18 ±2.74	79.12 ±1.25	69.14 ±0.99	66.14± 4.78
EAL(500mg/kg)^{ns}	50.12 ±2.56	60.17± 2.44	65.12 ±3.66	58.14 ±1.72	52.14 ±1.08

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 8: Effect of *E. agallocha* leaves extract on Uric acid

Group/Treatment	URIC ACID				
	0 week	4 week	8 week	12 week	16 week
Control	1.90 ±0.13	2.01 ±1.25	1.99 ±2.15	2.00 ±1.25	2.15 ±1.25
Diabetic^{ns}	1.99 ±0.12	0.99 ±0.24	0.65 ±2.08	0.68 ±1.23	0.87 ±1.92
EAL (250mg/kg)*	1.65 ±1.3	1.51± 0.25	1.25± 1.30	1.38 ±1.28	1.25 ±1.39
EAL(500mg/kg) ^{ns}	1.88 ±1.05	2.00 ±1.85	1.89 ±1.25	1.89 ±1.34	2.12 ±0.99

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 9: Effect of *E. agallocha* leaves extract on Creatinine

Group/Treatment	Creatinine				
	0 week	4 week	8 week	12 week	16 week
Control	0.52± 0.15	1.23± 0.75	0.99 ±1.28	1.00± 0.25	0.85 ±1.21
Diabetic ^{ns}	0.65 ±1.25	2.12 ±0.28	0.31± 0.98	0.54 ±1.23	0.21 ±0.25
EAL (250mg/kg) ^{ns}	0.65± 1.35	1.99 ±1.23	0.65 ±1.23	0.98 ±2.35	0.65 ±1.28
EAL(500mg/kg) ^{ns}	0.77 ±1.37	1.35± 0.99	1.21 ±1.01	1.00 ±1.09	0.99 ±1.24

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 10: Effect of *E. agallocha* leaves extract on Total protein

Group/Treatment	Total Protein				
	0 week	4 week	8 week	12 week	16 week
Control	1.21 ±0.12	5.54 ±1.23	6.12 ±1.04	8.52 ±1.25	7.99 ±1.52
Diabetic ^{ns}	1.23 ±0.35	2.01 ±1.01	4.12 ±2.10	3.18 ±0.17	2.53 ±1.68
EAL (250mg/kg) ^{ns}	1.61± 0.58	4.23 ±1.26	5.12 ±1.28	4.45 ±1.23	5.11 ±2.58
EAL(500mg/kg) ^{ns}	1.45 ±1.23	6.12 ±1.25	7.14 ±1.25	7.54 ±1.28	8.00 ±1.28

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 11: Effect of *E. agallocha* leaves extract on Blood sugar

Group/Treatment	Blood Sugar				
	0 week	4 week	8 week	12 week	16 week
Control	86.17± 1.31	89.12± 1.26	90.15± 2.87	89.32 ±1.27	90.12 ±1.27
Diabetic*	89.12 ±2.13	250.12± 1.87	264.14± 2.25	271.12± 1.25	287.18 ±1.34
EAL (250mg/kg)*	82.13 ±1.23	180.25 ±1.25	165.25 ±2.58	175.24± 1.28	165.12± 2.36
EAL(500mg/kg)*	87.15 ±1.23	135.41±1.02	123.25 ±1.28	118.28± 1.27	115.23± 0.39

All values are mean ±S.D. (n=10). $P < 0.05$ all groups are compared with control group (One-way ANOVA followed by Dunnett's multiple comparison test)

Table 12: Effect of *E. agallocha* leaves extract on antioxidant activity in Kidney tissues

Parameters	Control	Diabetic	EAL 250 mg/kg b.w.	EAL 500 mg/kg b.w.
S O D(units of activity/mg protein)	18.75±2.14	9.44±1.87	13.5±2.22	17.14±2.3
M D A(μM/mg protein)	45.17±3.33	99.53±4.47	66.51±2.55	55.27±3.28
CATALASE(micromoles of H_2O_2 decomposed/mg protein/min)	34.52±2.40	22.6±3.55	29.2±2.38	32.93±2.7
GSH(nM/mg protein)	30.45±2.7	12.80±3.3	26.40±2.9	27.90±2.4

Table 13: Effect of *E. agallocha* leaves extract on antioxidant activity in Heart tissues

Parameters	Control	Diabetic	EAL 250 mg/kg b.w.	EAL 500 mg/kg b.w.
S O D(units of activity/mg protein)	18.01±2.23	8.99±1.07	13.54±2.17	17.01±2.39
M D A(μM/mg protein)	46.99±3.13	99.50±3.47	65.51±2.15	54.99±3.28
CATALASE(micromoles of H_2O_2 decomposed/mg protein/min)	34.02±2.14	22.67±3.55	28.92±2.18	32.93±2.79
GSH(nM/mg protein)	30.33±2.7	12.70±3.39	25.49±2.9	26.97±2.14

Table 14: Effect of *E. agallocha* leaves extract on antioxidant activity in Liver tissues

Parameters	Control	Diabetic	EAL 250 mg/kg b.w.	EAL 500 mg/kg b.w.
S O D(units of activity/mg protein)	18.13±2.19	9.04±1.28	13.05±2.02	17.98±2.13
M D A(μM/mg protein)	44.17±3.01	98.30±4.43	66.20±2.05	55.07±3.98
CATALASE(micromoles of H_2O_2 decomposed/mg protein/min)	34.00±2.49	22.08±3.17	29.2±2.07	32.03±2.17
GSH(nM/mg protein)	30.45±2.78	12.10±3.83	26.49±2.19	28.90±2.41

Histopathology

Structural Changes in Kidney tissues: The histopathological examination of kidney in diabetic rats showed a severe tubular necrosis and degeneration. Treatment with higher dose of (500 mg/kg) EAL extract showed regeneration of tissue similar to the normal architecture of kidney, which indicates that the plant extract possesses nephron protective activity (Figure 1).

Structural Changes in Liver tissues: The histopathological examination of liver in diabetic rats showed a marked degeneration of the liver parenchyma correlated. Treatment with higher dose of (500 mg/kg) EAL extract improved the cytoarchitecture, with visible central veins surrounded by hepatocytes and well-arranged hepatic ducts, which indicates that the plant extract possesses hepatoprotective activity (Figure 2).

Structural Changes in Heart tissues: Photomicrographs of histopathological examination (10X) of the heart from control and experimental groups Section of the heart from control group shows normal architecture. Section of the heart from diabetic group reveals thrombus formation, contraction band necrosis, and inflammation. Sections of heart from EAL 250 & 500 mg/kg groups were shows normal architecture heart comparable to control group (Figure 3).

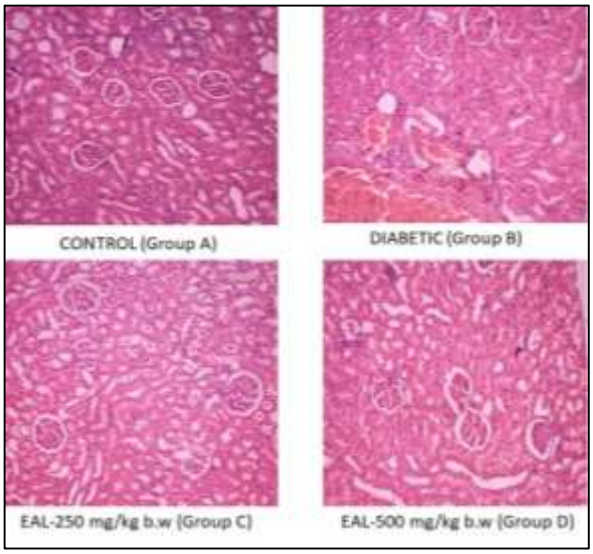

Fig 1: Structural Changes in Kidney tissues

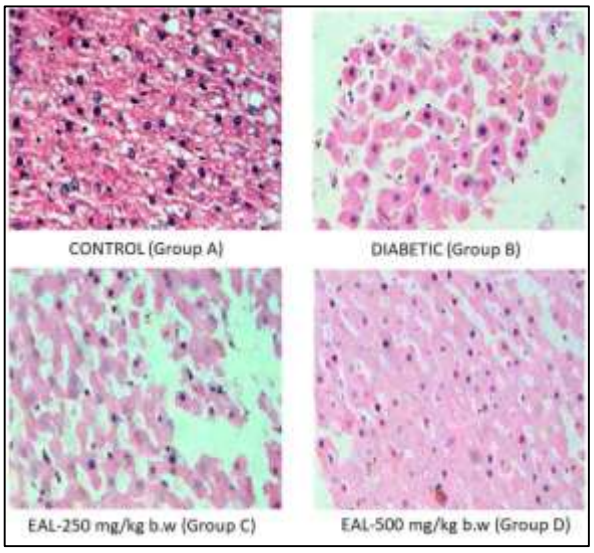

Fig 2: Structural Changes in Liver tissues

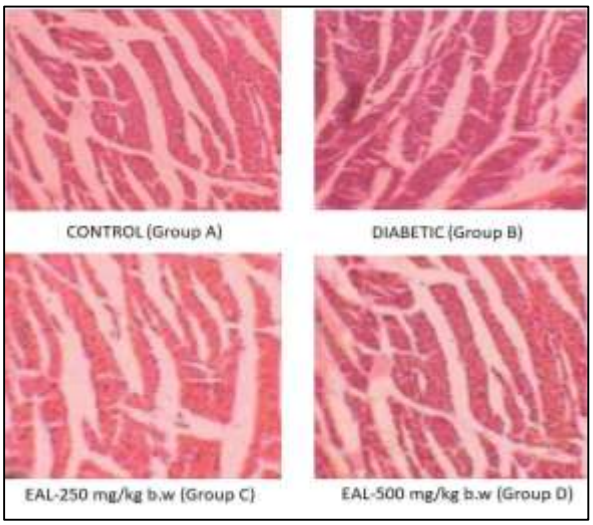

Fig 3: Structural Changes in Heart tissues

Discussion

In the present study, administration of single injection of streptozotocin (STZ) at a dose of 60 mg/kg, i.p. to rats resulted in development of oxidative stress damage in heart, hepatic and renal tissues. Diabetic group rats shows significant increase in the SGOT, SGPT, CK, serum urea and creatinine concentrations in the Group B rats when compared to the normal group (Group A).

Oral administration of ethanolic extract of *Excoecaria agallocha* L. significantly decreased in group C & D when compared to the Group B. The level of uric acid & total protein is significantly decreased in the Group B rats when compared to Group A. Oral administration of plant extract significantly increases the uric acid & total protein level in Group C & D when compared to the Group B. Thus, oxidative stress and lipid peroxidation are early events related to radicals generated during the hepatic metabolism of STZ [24]. Also, the generation of reactive oxygen species has been proposed as a mechanism by which many chemicals can induce nephrotoxicity [25, 26]. STZ induced oxidative stress results in lipoperoxidation, protein thiol oxidation, mitochondrial endoplasmic reticulum injury, altered homeostasis and irreversible DNA damage characterized by protein adduct formation [27, 28]. In recent studies have clearly shown that STZ increases the lipid peroxidation and suppresses the antioxidant defense mechanisms in renal tissue [29]. In the diabetic group animals, the MDA levels are increased significantly, when compared to normal control rats. On Administration of ethanolic extract of *Excoecaria agallocha* L., the levels of MDA decreased significantly when compared to diabetic group rats.

During diabetic induced complications superoxide radicals are generated at the site of damage and modulate SOD and CAT, resulting in the loss of activity and accumulation of superoxide radical, which increases oxidative stress and damages major organs in the body. SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism [30, 31]. The present study also demonstrated that STZ resulted in a decrease in the SOD, CAT activities when compared with normal control rats. It is due to enhanced lipid peroxidation or inactivation of the antioxidative enzymes. When rat is treated with the *Excoecaria agallocha* L. extract the reduction of SOD, CAT activity is increased significantly when compared with induced group B. Intracellular GSH plays an essential role in

detoxification of STZ and prevention of STZ toxicity [32]. The generation of the reactive oxygen species appears as an early event which precedes intracellular GSH depletion and cell damage. STZ also caused a significant decrease in GSH content. Administration of *Excoecaria agallocha* L. extract helped to uplift the GSH depletion induced by STZ.

In addition, alkaloids have also been reported to strongly inhibit lipid peroxidation induced in isolated tissues via its antioxidant activity. The protection offered by the extract could have been due to the presence of flavonoids and alkaloids. The leaves of the plant have rich in polyphenols and flavonoids due to the presence of these constituents the plant has antioxidant property. Diabetes mellitus is usually conveyed by excessive production of free radicals, hyperglycemia-induced mitochondrial reactive oxygen species production could be a key episode in the progress of diabetic complications [33, 34]. The antioxidant property of the plant has deteriorates the progression of diabetic-induced complications. The activity elicited by the extract might be due to its ability to activate antioxidant enzymes. The findings suggest the potential use of the ethanolic extract of *Excoecaria agallocha* L. as a novel therapeutically useful nephron protective, hepatoprotective and cardio protective agent.

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