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Research Article

ANTIDIABETIC, ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITIES OF THE ETHANOLIC EXTRACT OF THE LEAVES OF DIOSPYROS PILOSANTHERA BLANCO (FAMILY: EBENACEAE) AND ITS PREFORMULATION DEVELOPMENT

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ABSTRACT

Diabetes and liver cancer are among the leading causes of death worldwide. Diospyros species has been reported to have pharmacological applications arising from its extensive folkloric uses. The claims include anti-diabetic, antioxidant and antitumor properties. This study was carried out to investigate the anti-diabetic, antioxidant and hepatoprotective properties of D. pilosanthera, an endemic Philippine plant, and do a preformulation study.

Diabetes was induced by a single intra-peritoneal dose of streptozocin (45 mg/kg body weight [BW]). The extract (200, 500 and 1000 mg/kg BW) and glibenclamide (control, 600 µg/kg BW) were administered orally to diabetic Sprague-Dawley rats. Moreover, hepatoprotective property of the extract was evaluated against Diethylnitrosoamine (DENA) induced liver toxicity in rats. Rats were pre-administered orally with the ethanolic extract (200, 500 and 1000 mg/kg BW) and sillymarin 125 mg/kg BW) for fifteen days prior to a single dose of DENA (50 mg/kg BW; p.o.). In-vivo biochemical parameters like blood glucose, catalase, glutathione, alanine aminotranferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were evaluated to determine the antidiabetic, antioxidant and hepatoprotective activities of Diospyros pilosanthera. Histopathological examination of the liver tissues of the DENA-induced liver carcinogenic rats was also done. Pre-formulation study was also conducted where extract and common tablet excipients in a ratio of 1:1 were stored at room temperature and at 40°C for 60 days. The formulations were examined for compatibility and stability using organoleptic test.

The in-vivo antidiabetic and antioxidant study showed that the extract at 1000 mg/kg was the most effective dose, and was comparable with glibenclamide in lowering blood glucose level. It also prevented significant decrease in endogenous hepatic reduced glutathione and catalase levels. Evaluation of the hepatoprotective property of the extract revealed that a dose of 1000 mg/kg possessed significant hepatoprotective activity comparable with sillymarin. Treatment with the extract markedly obviated increases in ALT, AST and ALP while averting significant decreases in reduced glutathione and catalase enzyme levels. Histopathological changes such as centrilobular necrosis, extensive hepatocyte swelling and sinusoidal congestion with red blood cells were averted by extract administration. Phytochemical analysis showed the presence of alkaloids, flavonoids, tannins, glycosides, triterpenes and phenolic compounds. HPLC showed the probable presence of rutin that could be responsible for the tested pharmacological properties. Results of organoleptic examination showed that degradation of the extract was time and temperature-dependent. Degradation of the extract was observed after two months of storage at 40°C. The ethanolic extract was photosensitive and hygroscopic. Thus, it should be stored in a tightly sealed amber glass container at a temperature not higher than 30°C.

Results of the studies indicate that the ethanolic extract exhibited significant antidiabetic, antioxidant and hepatoprotective activities. The preformulation study revealed that the stability of ethanolic extract is time and temperature dependent.

Keywords: Hepatoprotective, antidiabetic, carcinogenesis, phytomedicine

INTRODUCTION

Diabetes and liver cancer are among the leading causes of death worldwide. Diabetes being the seventh leading cause of death[1], while Hepatocarcinoma is the fifth most common cancer, and that majority of patients with this type of cancer will die within one year[2].

Free radicals have been claimed to play an important role in affecting human health by causing several chronic diseases such as cancer, diabetes, aging, atherosclerosis, hypertension, heart attack and other degenerative diseases[3]. Various studies have shown that diabetes mellitus is associated with

increased formation of free radicals and decrease in antioxidant potential. Due to these events, the balance normally present in cells between formation and protection against them is disturbed. This leads to oxidative damage of cell components such as proteins, lipids and nucleic acid[4]. While hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages[5].

Diospyros species has been reported to have pharmacological applications arising from its extensive folkloric uses. The claims include anti-diabetic, antioxidant and antitumor properties. Among these are Diospyros cordifolia which at doses of 25 and 50 mg/kg BW significantly normalized the elevated blood glucose level and restored serum and biochemical parameters of STZ-induced diabetic rats towards normal values[6], and D. lotus aqueous extract administered at doses of 1000 and 1500 mg/kg BW also caused significant decrease in glucose level in STZ-induced diabetic rats[7], D. malabarica[8] and D. preussi[9] were proven to possess antioxidant activity and D. lotus[10] and D. montana[11] extract showed cytoxicity against cancer cell lines.

Despite the numerous proven therapeutic effects of Diospyros species and the availability of D. pilosanthera in the Philippines, its use is limited only in furniture industry. The possible reason for this is the lack of preformulation and formulation studies of the crude drug and crude extract of D. pilosanthera, which could facilitate its development into a useful drug product.

Preformulation is a developmental stage, which involves the characterization of the physical and chemical properties of a drug material for the purpose of making a stable, effective and safe dosage form. It also involves the application of biopharmaceutical principles to the physicochemical parameters of a drug with the goal of designing an optimum drug delivery system[12].

Insufficient preformulation studies on a certain drug will lead to unstable, ineffective and unsafe dosage formulations. The study therefore, hopes to provide critical information on the use of a crude extract for the preparation of a stable, safe, and effective formulation. Thus, this study was carried out to investigate the anti-diabetic, antioxidant and hepatoprotective properties of D. pilosanthera, an endemic Philippine plant, and to do a preformulation study.

MATERIALS AND METHODS

Plant Material

The leaves of D. pilosanthera were collected from Victoria, Mindoro Oriental in April 2012. The collected specimen was submitted to the National Museum for authentication. A herbarium of the plant was also submitted and kept at the UST Herbarium Center.

Drugs and Chemicals

Analytical grade of ethanol (99%) used for the extraction of the leaves was procured from Bellman Corp. Chemicals used to induce diabetes and liver carcinogenesis like Streptozocin and DENA, as well as reagents to test for catalase and reduced glutathione activities were procured from Sigma-Aldrich and Merck Incorporated. Positive controls like glibenclamide and Sillymarin were also bought from the same companies. All other unstated chemicals and reagents were of analytical grade.

Animals

Male and female Sprague Dawley rats weighing 130-180 grams purchased from the Federal Drug Administration (FDA), Philippines were used. Animals were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Santo Tomas and was in accordance with their guidelines. The animals were fed ad libitum with normal laboratory pellet diet and water. Animals were maintained under a constant 12 hour light and dark cycle and at environmental temperature of 210C-230C. The animals were housed at the animal laboratory facility of the University of Santo Tomas Research Center for the Natural Sciences.

High Performance Liquid Chromatography

HPLC analysis was conducted using Agilent 1200 series HPLC system equipped with quaternary pump and autosampler. Separation of the compounds was effected through a C-18 reverse phase column of an Agilent Series II-HPLC machine at 300C. The solvent system was composed of ethanol:phosphoric acid. For identification of rutin, forty milligram of sample was diluted with 5 mL ethanol. The sample was subjected to gradient elution with ethanol:0.5% phosphoric acid in water (50:50) at a flow rate of 0.9 mL/min and detection at 365 nm with a total run time of 40 minutes. The sample was subjected to gradient elution with

ethanol: 0.2% phosphoric acid in water (65:35) at a flow rate of 1 mL/min and detection at 365 nm with a total run time of 45 minutes.

Approximate Lethal Dose Determination

According to the Organization for Economic Cooperation and Development (OECD), a limit test is used when an extract is either known or expected to be practically non-toxic. The limit test was carried out using five female Sprague-Dawley rats. One rat was given 2000 mg/kg of extract via oral gavage and was observed continuously for 2 hours for behavioral, neurological and autonomic profile and after a period of 24 hours, for any lethality, moribund state or death. The rat survived hence the rest of the four rats were given the same dose of the extract and observed for a 14 day period. Since none of the rats died, they were all sacrificed via cervical dislocation and disposed properly. All animal testings done in the research followed the guidelines provided by OECD and under protocol number AR-2012-147 issued by the Institutional Animal Care and Use Committee of the University of Santo Tomas, Research Center for the Natural Sciences.

Induction of Experimental Diabetes

Twelve (12) hour fasted experimental animals (24 rats) received freshly prepared solution of streptozocin (STZ) (45 mg/kg) in 0.1 M sodium citrate buffer (1 mL/kg BW) by a single intraperitoneal injection (Siddique et al, 1987). While normal rats (6 rats) received 1 mL/kg BW citrate buffer as vehicle. Two days after STZ administration. The rats were fasted overnight and 1 ml of blood sample was collected from the tail vein. Rats showing moderate diabetes with glycosuria and hyperglycemia (with blood glucose levels of 200 mg/dL and above) were used for the experiment.

Experimental Design to test for the Antidiabetic activity

A total of 36 rats (30 diabetic rats and 6 normal rats) were divided into 6 groups. All treatments were administered daily by oral gavage for 15 days.

Group 1: Normal rats were given 2 mL/kg BW distilled water (normal control)

Group 2: Diabetic control rats were given 2 mL/kg BW of distilled water (diabetic control)

Group 3: Diabetic rats were given 200 mg/kg ethanolic extract solution

Group 4: Diabetic rats were given 500 mg/kg ethanolic extract solution

Group 5: Diabetic rats were given 1000 mg/kg ethanolic extract

Group 6: Diabetic rats were given 600 ug/kg glibenclamide

Sample Collection

Animals from each group were deprived of food overnight but with free access of water before taking blood samples via tail clippings. Blood Glucose level using Glucose assay kit. Blood glucose estimations were done after STZ induction and at 3, 6, 10 and 15 days post treatment. Weight was also determined on 0 and 15 days. Following blood collection on the 15th day, all rats were sacrificed by cervical dislocation and the livers were excised. Livers of the experimental rats were collected and prepared as homogenates for the determination of antioxidant activity using catalase and reduced glutathione assays.

Experimental Design to test for the Hepatoprotective activity

Thirty six (36) Sprague-Dawley rats (3 males, 3 females) divided into 6 groups of 6 rats each were treated by oral gavage. Treatment consisted of pretreatment phase of 0.9% normal saline as the (normal control), ethanolic extract of D. pilosanthera and silymarin (positive control) for 14 days followed by the second phase in which rats from Groups 2-6 were given 50 mg/kg of Diethylnitrosoamine (DENA) as the toxicant on day 15. All test substances were dissolved in 0.9% normal saline solution. Blood was extracted via tail clipping and the serum was separated from the collected blood. Blood (serum) collected before administration of any test substance and toxicant (day 0) were used for baseline values whereas those collected at the end of postreatment of the toxicant (day 18) were used for endpoint data for different biochemical parameters of hepatoxicity and liver function test. Following blood collection on the 18th day (4 days after intoxicated with DENA), all rats were sacrificed via cervical dislocation and the liver was excised. A major portion of the liver was preserved in 10% formalin for histopathological analysis while the remaining portion was used to prepare the liver homogenate for antioxidant enzyme assays like glutathione and catalase.

Group 1: served as control (normal untreated rat) received 2 mL/kg BW of .9% normal saline.

Group 2: pretreated with 2ml/kg BW of 0.9% normal saline solution (DENA control)

Group 3: pretreated with 200 mg/kg BW D. pilosanthera extract solution

Group 4: pretreated with 500 mg/kg BW D. pilosanthera extract solution

Group 5: pretreated with 1000 mg/kg BW D. pilosanthera extract solution

Group 6: pretreated with 125 mg/kg BW Sillymarin

Hepatoprotective Assessment

The serum collected from each rat during the course of the experiment was used to measure certain biochemical parameters that are indicative of liver damage (the transaminases- ALT and AST) and altered liver function (alkaline phosphatase). Standard kits purchased from Human Diagnostics (Human Gmbh) were used. Tests were done in triplicate.

Alanine Aminotransferase Assay

A standard kit containing a buffer and a substrate, was used to measure ALT. The buffer consists of L-alanine (625 mM) and Lactate dehydrogenase/LDH (1.5 kU/l) while the substrate consists of 2-oxoglutarate (75 mM) and NADH (0.9 mM). To assay for ALT, 10 µL of sample was mixed with 100 µL of buffer. The mixture was incubated for five mins at 370C and added with 25 µL of substrate. The absorbance was taken at 340 nm every minute for three minutes.

Aspartate Aminotransferase Assay

A standard kit was used. The kit is composed of a buffer containing L-aspartate (300mM), lactate dehydrogenase (1.13 kU/l) and malate dehydrogenase (0.75 kU/l) as well as a substrate composed of 2-oxoglutarate (60mM) and NADH (0.9mM). For the assay, 10 µL of sample was mixed with 100 µL of buffer and incubated at 370C for five minutes. The mixture was added with 25 µL of substrate, incubated again for one minute and the absorbance was read at 340 nm every minute for 3 minutes.

Alkaline Phosphatase Assay

A kit (Alkaline phosphatase liquicolor) containing a buffer and substrate was used to measure ALP activity. The buffer is mainly composed of diethanolamine (1.25 M pH 10.35) and magnesium chloride (0.625 mM) while the substrate is composed of p-Nitrophenylphosphate (50 mM). To assay for ALP activity (U/l), 2 µL of the sample was combined with 100 µL of buffer then incubated for 1 minute at 370C. The mixture was then added with 25 µL of substrate and incubated again for another minute. Absorbance is read at 404 nm per minute for 3 minutes.

Estimation of Anti-oxidant Activity

Preparation of Liver Homogenate

Liver homogenate was prepared by adding 1 mM EDTA to the liver using a homogenizer. The unbroken cells and debris were removed by centrifugation at 10,000 rpm for 15 minutes at 40C using a cooling centrifuge and the supernatant liquid were used.

Catalase Activity

An aliquot of supernatant (10 µL) was pipette into a microplate and reaction was started by the addition of 100 µL freshly prepared H2O2 (19mM) in phosphate buffer, and 195 µL of Phosphate buffer (50mM, pH 7.4). The rate of H2O2 decomposition was measured at 240 nm per minute and catalase activity in U/l was calculated using the molar absorption coefficient of H2O2 (43.6 M-1cm-1) at 240 nm.

Reduced Glutathione

An aliquot of 0.5 mL supernatant from the homogenate was precipitated with sulphosalicylic acid (0.5 mL, 4% w/v). The mixture was kept at a temperature of at least 4 C for one hour then centrifuged for 15 minutes at 1200 x g. A 10 µL aliquot from the supernatant of the centrifuged mixture was taken and added with 270 µL of phosphate buffer (50nM, pH 7) and 20 µL of 5',5-Dithiobis-2-nitrobenzoic acid or DTNB (4.5 mM). A blank was used containing phosphate buffer only. The absorbance was read at 412 nm and the concentration of reduced glutathione (µmol/g sx) was computed from the molar absorption coefficient (€) of DTNB (14,150 M-1cm-1 at 412 nm).

Histopathological Analysis

Liver samples were preserved in 10% formalin and taken to the Histopathology Department of the Philippine Kidney Dialysis Foundation (PKDF) for slide preparation. The samples were dehydrated in ascending grades of alcohol and embedded in melted hard paraffin then allowed to solidify at room temperature forming blocks. The paraffin blocks were cut into five micron thickness and mounted on clean glass slides then stained with hematoxylin and eosin (H and E) to visualize the general morphology of the tissue sample. The slides were then taken to Dr. Kalangitan

Gutierez, an experienced histopathologist working at the Veterans Memorial Medical Center, for reading and interpretation to assess the level of NDEA hepatotoxicity. The slides were examined further using an Electron Microscope at the Histopathology Section of Veterans Hospital. Histology was taken as end point biomarkers.

PREFORMULATION STUDIES

Drug- Excipient Compatibility Testing

The powdered ethanolic extract was mixed with several excipients which included starch, lactose, talc and magnesium stearate, which are commonly used in the manufacture of tablets. The formulation consisted of 1:1 ratio of crude extract and excipient . The drug-excipient formulations were placed in air tight, amber containers and stored at 30 ± 2 °C (room temperature) and at accelerated temperature using stability oven maintained at 40 ± 2°C for 60 days. Chemical incompatibility of the solid extract-excipient combination indicative of degradation was determined by organoleptic observation. Signs of decomposition like physical changes manifested by color, appearance, discoloration, formation of granules and hygroscopicity were observed and documented.

Statistical Analysis

The antidiabetic, antioxidant and hepatoprotective activities of the ethanolic extract were calculated using probit regression analysis. All assays were performed in triplicate. Experimental results were expressed as mean ± standard error (SEM). Independent t-test and one-way analysis of variance (ANOVA) were used to compare two or more groups of data. Post hoc analysis using Tukey and LSD were used with ANOVA to find any significant difference/s between groups. p values of less than 0.05 were considered significant. SPSS software version 19 was used for statistical analysis.

RESULTS

Extraction

Percolation of the ground Diospyros pilosanthera leaves using 99% ethanol yielded 27% of the dry extract. The extract was dark green in color with a strong leafy odor and a pasty consistency which dries up when stored at 20C-80C. Further lyophilization of the ethanolic extract yielded a powdered extract.

Toxicity Test (Limit Test)

The toxicity test prescribed by the Organization for Economic Cooperation and Development (OECD) is a simple test used to estimate the lethal dose (LD50) of a certain chemical like a plant extract. A limit test is used when an extract is either known or expected to be practically non-toxic (OECD, 2008). Based on previous studies of Diospyros species, the leaves are macerated or prepared as a decoction and taken orally with no toxic effects. Further, fruit D. pilosanthera has been reported to be edible13. Hence, the limit test dose of 2000 mg/kg was administered to five (5) Sprague-Dawley rats. No overt signs of clinical toxicity were observed from all the rats during the 14-day period.

Phytochemical Screening

Phytochemical screening of the extract was carried out at the Industrial Pharmacy laboratory of the University of the Philippines, Manila. Results in table 1 showed the presence of glycoside, tannins, phenols, alkaloids, triterpenes, flavonoids and sugars.

High Performance Liquid Chromatography

Chromatographic profiles of the fraction of the extract and rutin is shown in figures 1 and 2. The presence of rutin, a potent anti-oxidant flavonoid was identified in the diluted ethanolic extract of D. pilosanthera.

Antidiabetic Activity

Effect of D. pilosanthera on Weight of Experimental Rats (Table 2) shows that the body weights of rats from diabetic control group (after 15 day treatment) were significantly decreased compared with normal group. Administration of D.pilosanthera extract significantly increased the body weights towards normal in a dose dependent manner.

Blood Glucose Level

The Fasting blood glucose (FBG) levels of normal, diabetic and treated rats are summarized in table 3. STZ at the dose of 45 mg/kg produced marked hyperglycemia as evident from significant elevation in FBG level in dibatic control group as compared to normal group. Administration of D. pilosanthera extract (15-day treatment) in STZ-induced diabetic rats at the dose of 1000 mg/kg caused the fall in blood glucose levels to normal comparable to the normal control and Glibenclamide groups.

Table 1. Constituents present in the extract

Constituents	Name of Test/Reagents	Theoretical Results	Actual Results	Indication
Glycosides	Lead acetate	White Precipitate	White precipitate (+)	Presence of glycosides
Tannins	Ferric chloride Test	Bluish- black	Bluish-black coloration	Presence of tannins and Phenolic groups
Alkaloids	Mayer's	Yellow cream ppt	Yellow cream ppt (+++)	Presence of alkaloids
	Dragendorff's	red ppt	Red-orange ppt (++)	
	Valser's	w/ precipitation	white ppt (+++)	
	Hager's	yellow ppt	yellow ppt (+++)	
	Wagner's	reddish-brown ppt	reddish-brown ppt(+++)	
Triterpenes Sterols	Liebermann-Burchard Test	Green to red coloration indicates triterpenes Blue coloration indicates sterols	Green to red discoloration	Presence of Triterpenes
Saponins	Froth Test	Honeycomb froth	No honeycomb froth	Absence of saponins
Flavonoids	Wilstatter "Cyanidin" Test	Orange to red color	Pink solution	Presence of flavonoids
Sugar	Fehling's test	Brick red ppt.	Brick red ppt	Presence of sugars
Plant acids	Sodium Carbonate test	Evolution of gas	No evolution of gas	Absence of plant acids

Table 2. Mean Weights (g) of Diabetic Rats at Day 0 and 15 Day-Post Treatment

Group	Day 0	Day 18	% increase	*F* stat	*p*-value
[A]normal rats + vehicle	178.81 ± 3.75	*203.95 ± 3.02	14.20 ± 1.82	90.045	<0.001
[B]diabetic rats + vehicle	174.23 ± 2.37	**143.72 ± 2.00	-17.48 ± 1.16		
[C]diabetic rats + 200 mg/kg extract	170.83 ± 5.82	**160.59 ± 5.15	-5.89 ± 1.77		
[D]diabetic rats + 500 mg/kg extract	159.23 ± 3.10	*171.05 ± 2.73	7.51 ± 1.51		
[E]diabetic rats + 1000 mg/kg extract	166.80 ± 1.36	*194.79 ± 1.23	16.81 ± 1.08		
[F]diabetic rats+ Glibenclamide	168.80 ± 3.48	*195.95 ± 3.77	16.14 ± 1.34		

Values expressed as mean ± SEM, n = 6

* $p<0.05$ significant increase in weight from day 0 to day 18

** $p<0.05$ significant decrease in weight from day 0 to day 18

The % increase of weights significantly differ: [$F_{5,30}$ = 90.045, $p<0.001$; post hoc: B<C<D<(A=E=F)].

Table 3. Blood Glucose Level (mg/dL) of the Diabetic Rats at 15 day- Post Treatment

Group	Baseline	3 days after STREP	post txt 3 days	post txt 6 days	post txt 10 days	post txt 15 days	F stat	*p*-value
[A]normal-vehicle	96.01 ± 1.06	109.22 ± 3.11	100.62 ± 2.00	98.04 ± 1.69	94.97 ± 3.85	93.36 ± 2.23	$5.256_{4,20}$	0.005
[B]diabetic-vehicle	110.26 ± 1.89	471.63 ± 7.90	522.02 ± 30.53	619.50 ± 5.88	627.75 ± 6.26	619.04 ± 4.69	23.007	<0.001
[C]200 mg/Kg extract	97.94 ± 4.56	446.10 ± 6.58	477.75 ± 2.79	552.05 ± 5.76	555.24 ± 4.76	511.93 ± 6.28	80.220	<0.001
[D]500 mg/Kg extract	107.30 ± 4.40	436.08 ± 3.52	416.18 ± 7.45	420.36 ± 6.72	375.69 ± 6.08	313.65 ± 3.37	67.320	<0.001
[E]1000 mg/Kg extract	116.05 ± 5.29	451.15 ± 4.87	346.76 ± 6.24	255.58 ± 10.63	172.11 ± 7.97	114.71 ± 5.27	917.547	<0.001
[F]Glibenclamide	103.71 ± 5.29	447.86 ± 9.49	333.26 ± 6.96	248.07 ± 2.01	165.64 ± 6.14	109.11 ± 3.02	393.938	<0.001

Values expressed as mean ± SEM, n = 6.

Blood Glucose of the rats significantly differ [$F_{5,29}$= 2078.550, $p<0.001$; B<C<D<(A=E=F)]

Table 4. Reduced Glutathione Activity (µmol/g sx) of the Liver of Diabetic Rats after 15 day-post treatment

Group	Reduced Gluthatione Activity	F stat	*p*-value	Post hoc
[A]Normal	3.92 ± 0.09	22.332	<0.001	(B=C=D)<(A=E=F)
[B]Diabetic Rats + Distilled Water	1.16 ± 0.24			
[C]Diabetic Rats + 200 mg/Kg extract	1.41 ± 0.29			
[D]Diabetic Rats + 500 mg/Kg extract	2.07 ± 0.43			
[E]Diabetic Rats + 1000 mg/Kg extract	3.26 ± 0.17			
[F]Diabetic Rats + Gilbenclamide	3.49 ± 0.05			

Values expressed as mean ± SEM; n = 6

Table 5. Catalase Activity (U/mL) of the Liver of Diabetic Rats after 15 Day-Post Treatment

Group	Catalase Activity	F stat	*p*-value	Post hoc
[A]Normal rats + vehicle	43.02 ± 1.83	16.368	<0.001	(B=C)<D<(A=E =F)
[B]Diabetic rats + vehicle	20.17 ± 2.46			
[C]Diabetic Rats + 200 mg/Kg extract	26.39 ± 2.36			
[D]Diabetic Rats + 500 mg/Kg extract	32.96 ± 2.10			
[E]Diabetic Rats + 1000 mg/Kg extract	39.41 ± 2.22			
[F]Diabetic Rats + glibenclamide	41.27 ± 2.46			

Values expressed as mean ± SEM; n = 6

Table 6. Alanine Aminotransferase (ALT) Activity (U/L) of the Liver of Rats at Days 0 & 18 After Post Treatment with DENA

Group	Day 0	Day 18	t stat	*p*-value
[A]Vehicle	20.85 ± 0.82	21.21 ± 0.36	0.545	0.609
[B]Vehicle w/ DENA	19.95 ± 1.27	40.44 ± 1.36	8.920	<0.001
[C]200 mg/Kg extract w/ DENA	19.41 ± 0.56	35.59 ± 1.60	10.978	<0.001
[D]500 mg/Kg extract w/ DENA	19.59 ± 1.13	30.37 ± 2.78	3.641	0.015
[E]1000 mg/Kg extract w/ DENA	19.77 ± 0.72	22.82 ± 0.58	3.397	0.019
[F]Sillymarin w/ DENA	19.23 ± 0.94	21.03 ± 1.66	1.388	0.224

Values expressed as mean ± SEM, n = 6

ALT activity significantly differ after 15 day-post treatment: [$F_{5,30}$ = 26.370, p<0.001]; post hoc: [(A=E=F)<D< C<B]

Table 7 Aspartate Aminotransferase (AST) Activity (U/L) of the Liver of Rats at Days 0 & 18 after Post Treatment with DENA

Group	Day 0	Day 18	t stat	*p*-value
[A]Vehicle	40.15 ± 3.78	41.26 ± 2.28	0.276	0.794
[B]Vehicle w/ DENA	40.81 ± 2.59	67.29 ± 2.70	5.740	0.002
[C]200 mg/Kg extract w/ DENA	40.59 ± 2.12	59.12 ± 1.06	6.529	0.001
[D]500 mg/Kg extract w/ DENA	41.92 ± 2.64	56.70 ± 1.98	3.676	0.014
[E]1000 mg/Kg extract w/ DENA	40.37 ± 1.83	44.34 ± 1.27	3.505	0.017
[F]Sillymarin w/ DENA	41.03 ± 2.69	43.02 ± 2.55	0.423	0.690

Values expressed as mean ± SEM, n = 6

AST activity significantly differ after 18 days-post treatment: [$F_{5,30}$ = 26.370, p<0.001; post hoc: [(A=E=F)<D< C<B]

Table 8. Alkaline Phosphatase (ALP) Activity (U/L) of the Liver of Rats at Days 0 & 18 After Post Treatment with DENA

Group	Day 0	Day 18	t stat	*p*-value
[A]Vehicle	118.44 ± 2.88	119.58 ± 3.57	0.268	0.800
[B]Vehicle w/ DENA	119.58 ± 3.16	192.82 ± 6.47	9.914	<0.001
[C]200 mg/Kg extract w/ DENA	119.97 ± 6.39	171.84 ± 4.76	10.333	<0.001
[D]500 mg/Kg extract w/ DENA	120.16 ± 4.48	145.33 ± 6.52	4.945	0.004
[E]1000 mg/Kg extract w/ DENA	120.54 ± 4.80	124.74 ± 2.85	0.666	0.535
[F]Sillymarin w/ DENA	119.39 ± 2.28	121.87 ± 5.18	0.389	0.713

Values expressed as mean ± SEM, n = 6

Mean ALP after 18 days- post treatment significantly differ: [$F_{5,30}$= 35.651, p<0.001; post hoc: [(A=E=F)<D< C<B]

Table 9. Reduced Glutathione Activity (U/mL) of the Liver of Rats after 18 Day-Post Treatment with DENA

Group	Reduced Glutathione Activity	F stat	*p*-value	Post hoc
[A]Vehicle	3.79 ± 0.06	59.251	<0.001	(B=C)<D<(E=F)<A
[B]Vehicle w/ DENA	1.56 ± 0.08			
[C]200 mg/Kg extract w/ DENA	1.82 ± 0.22			
[D]500 mg/Kg extract w/ DENA	2.13 ± 0.12			
[E]1000 mg/Kg extract w/ DENA	3.14 ± 0.06			
[F]Sillymarin w/ DENA	3.28 ± 0.09			

Values expressed as mean ± SEM, n = 6

Table 10: Catalase Activity (U/mL) of the Liver of Rats after 18 Day-Post Treatment with DENA

Group	Catalase Activity	F stat	*p*-value	Post hoc
[A]Vehicle	48.97 ± 2.95	21.367	<0.001	B<C<D<(A=E=F)
[B]Vehicle w/ DENA	21.14 ± 3.11			
[C]200 mg/Kg extract w/ DENA	29.69 ± 2.60			
[D]500 mg/Kg extract w/ DENA	38.82 ± 1.39			
[E]1000 mg/Kg extract w/ DENA	46.75 ± 0.54			
[F]Sillymarin w/ DENA	47.34 ± 2.88			

Values expressed as mean ± SEM, n = 6

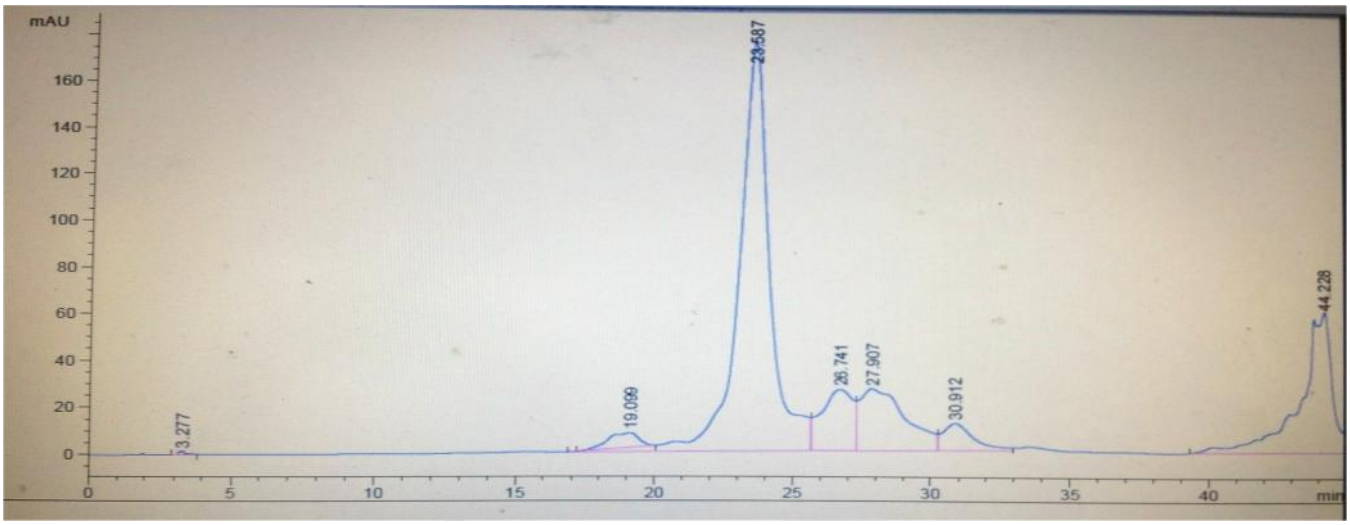

Figure 1. Chromatogram of D. pilosanthera extract at 365 nmshowing the peak retention time at 23.587 mins

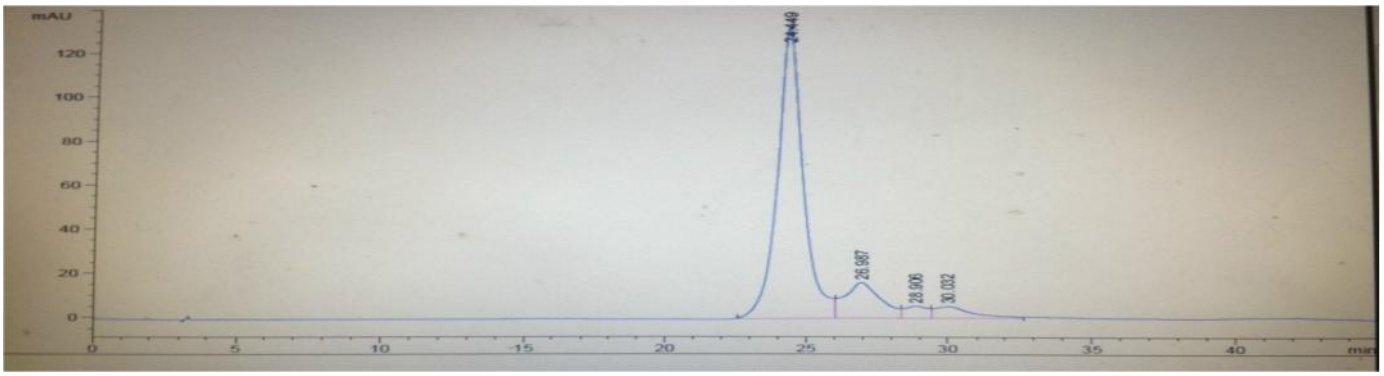

Figure 2. Chromatogram of Rutin at 365 nm showing peak retentiom time at 24.449 mins

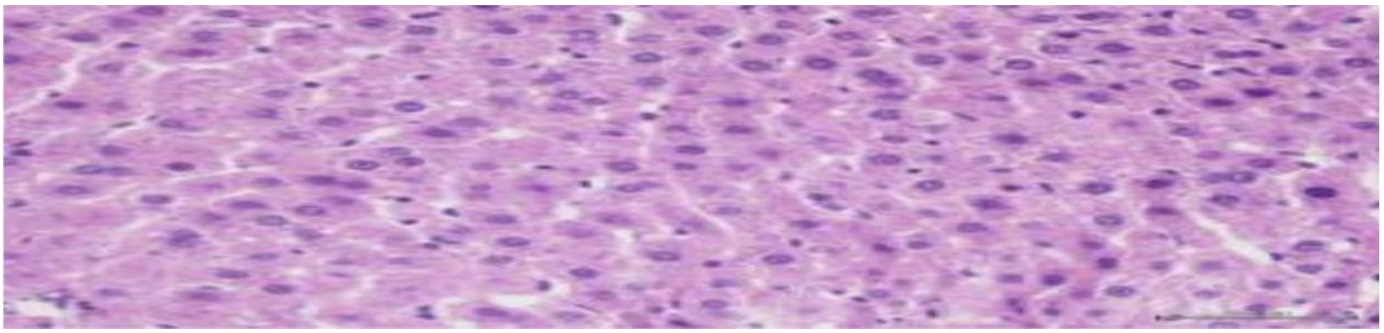

Figure 3. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the control group. Liver shows intact central vein surrounded by healthy hepatocytes with well preserved cytoplasm and prominent nucleus and nucleolus, and sinusoids between hepatocytes.

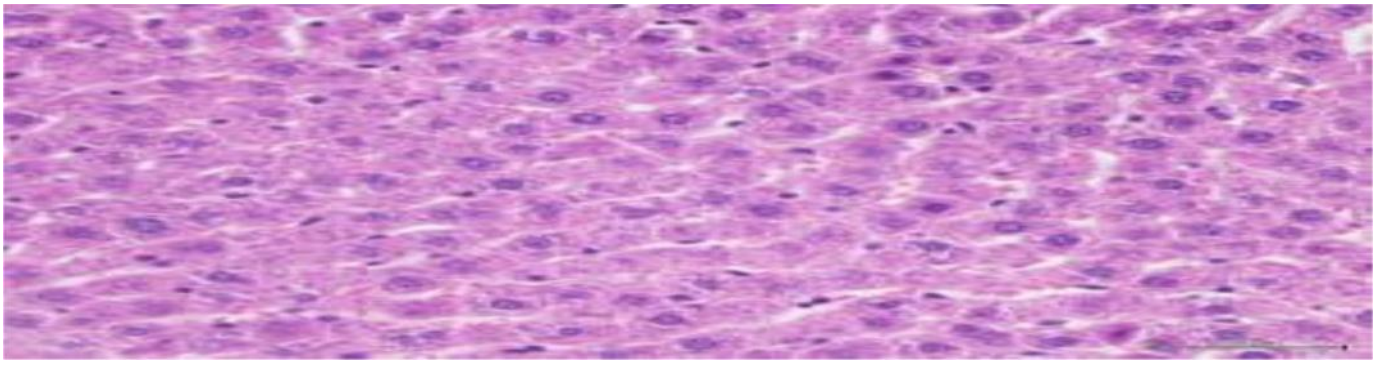

Figure 4. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the DENA toxicant group. Liver tissue reveals centrilobular necrosis, with blood pooling in sinusoidal spaces.

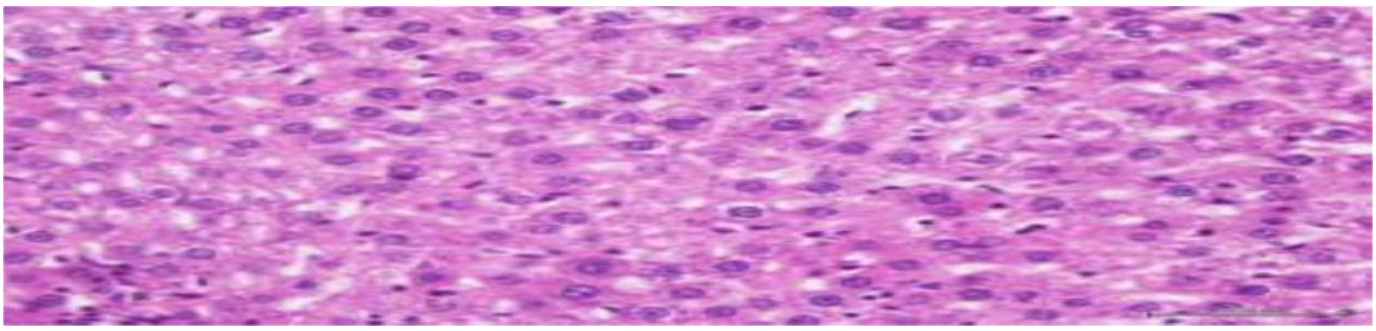

Figure 5. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the 200 mg/kg-dose extract treated group. Image shows cellular swelling and moderate scattered inflammatory cell infiltrates consisting of neutrophils, lymphocytes and Kupffer cells. Minimal necrosis is also observed.

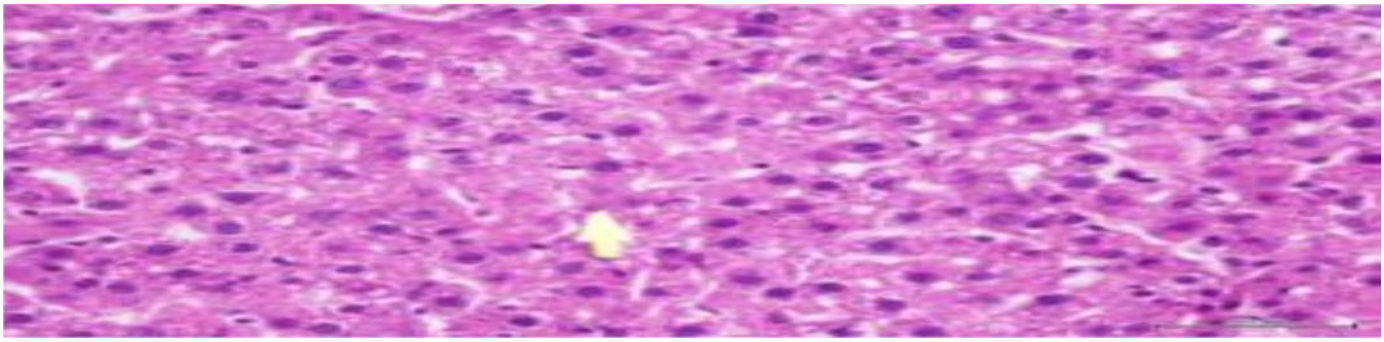

Figure 6. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the 500 mg/kg-dose extract treated group. Image shows scant necrosis (as pointed by the yellow arrow), cellular swelling and fewer scattered inflammatory cells.

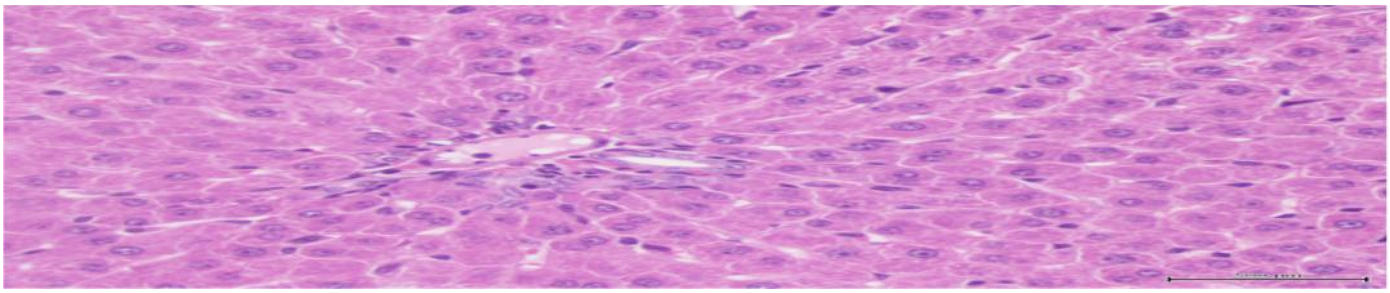

Figure 7. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the 1000 mg/kg-dose extract treated group. Image shows a central vein surrounded by normal hepatocytes. Regenerative changes are present as seen from the binucleated cells. Kupffer cells are also present.

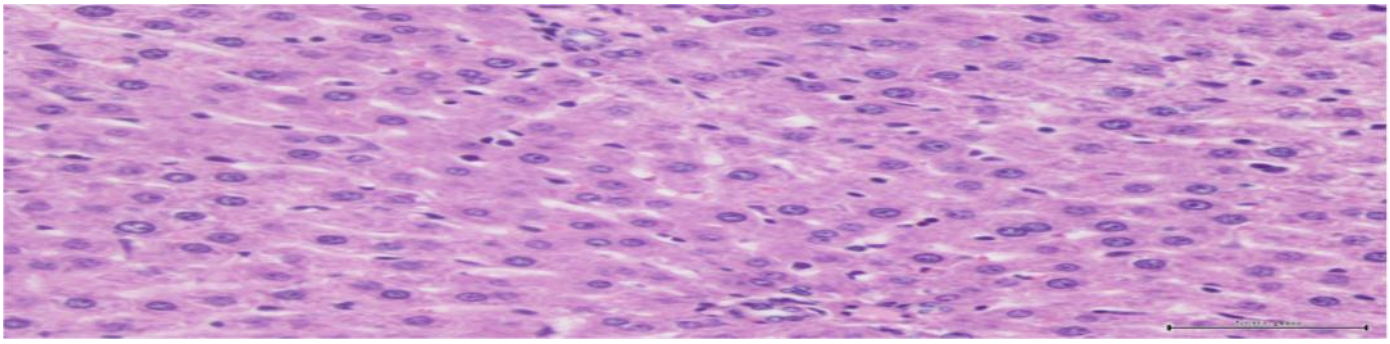

Figure 8. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the Sillymarin-dose extract treated group. A normal centrilobular vein is shown surrounded by normal hepatocytes. Regenerative changes are evident as shown by the binucleated cells. Kupffer cells are interspersed with hepatocytes around the area.

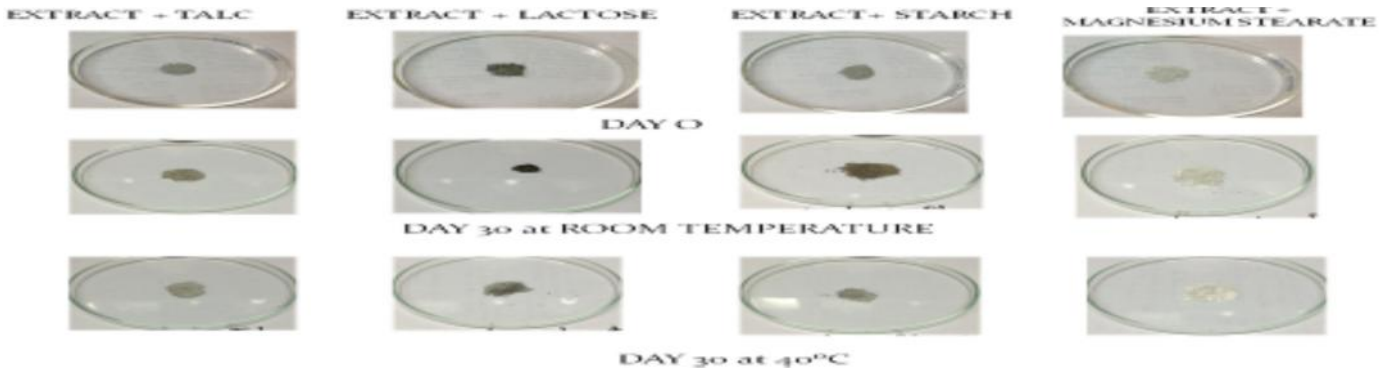

Figure 9. Stability testing of Excipient-extract at room temperature and at 40 degrees Celsius for 60-day storage period

Antioxidant Activity of Diabetic Rats

Reduced Glutathione and Catalase Assay

The level of reduced glutathione (GSH) and catalase activity were significantly depleted in diabetic control group as compared with normal group as shown in tables 4 qnd 5. Reduced GSH and CAT level were found to be significantly and dose dependently elevated towards normal level on administration of D. pilosanthera extract. Treatment with 1000 mg/kg of the extract recovered GSH and CAT activities towards normal levels comparable to normal control and Glibenclamide groups after 15 day treatment.

Hepatoprotective Activity

Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) Assay

Biochemical parameters like ALT, AST and ALP in DENA control group were significantly elevated (after 18 day post treatment) when compared to the normal control group. Treatment with D. pilosanthera extract at the dose of 1000 mg/kg significantly brought the ALT, AST and ALP levels towards the normal values comparable to the normal control and Sillymarin groups. Tables 6, 7 qnd 8 show the summary of results.

Reduced Glutahione and Catalase Assays

The level of reduced glutathione (GSH) and catalase activity were significantly depleted in the liver of DENA control group as compared with normal group as shown in tables 9 qnd 10. Reduced GSH and CAT level were found to be significantly and dose dependently elevated towards normal level on administration of D. pilosanthera extract. Treatment with 1000 mg/kg of the extract recovered GSH and CAT activities towards normal levels comparable to normal control and Glibenclamide groups after 18 day post treatment with DENA.

Histopathological Examination

Figure 3 shows a representative liver tissue from the control group. The administration of 2 ml/kg of distilled water showed normal hepatic lobule architecture with well arranged hepatocytes and no remarkable changes at the central vein. A contrasting image of hepatic architecture from the toxicant group is presented in figure 4. The liver tissue shows enlarged nuclei, disruption of the polyhedral shape of hepatocytes and abundant cytoplasm, indicative of cellular swelling. Pooling of red blood cells in the sinusoidal spaces is also observed along with cellular necrosis. A light microphotograph of a liver tissue from the 200 mg/kg-dose and 500 mg/kg-dose extract treated group is shown in Figures 5 and 6. Cellular swelling is evident and moderate scattered inflammatory cell infiltrates consisting of neutrophils, lymphocytes and Kupffer cells were noted in the lowest-dose treated group. Minimal cellullar necrosis was also observed. The liver tissue representing the 500 mg/kg-dose extract group showed a more enhanced liver architecture with scant cellular necrosis, fewer cellular swelling and lesser scattered inflammatory cell infiltrates consisting of neutrophils, lymphocytes and Kupffer cells. The 1000 mg/kg -dose extract treated group showed a better liver architecture which is comparable with Sillymarin-dose treated group as shown in Figures 7 and 8. A photomicrograph of a liver tissue from the highest-dose extract treated group reveals prominent nucleated hepatocytes surrounding the centrilobular region. Evidence of regeneration is present based on the bi-nucleated hepatocytes scattered inflammatory cells around the central vein, suggesting karyokinesis. The hepatocytes are arranged in cords and scant red blood cells. Kupffer cells are also noted. The hepatic architecture of the representative tissue sample from the Sillymarin-dose treated group was not different from the tissue sample of the highest-dose extract group. Light microscopy revealed mono and binucleated hepatocytes arranged in cords. Sinusoidal congestion of red blood cells was noted but unremarkable. The presence of Kupffer cells, which were mentioned previously to have a possible role in hepatocyte regeneration, as well as the presence of binucleated cells, may suggest regeneration of the hepatic cells.

Preformulation Study

Preformulation study is one of the important prerequisite in development of any drug delivery system.. Figure 9 shows that there were no caking, liquefication, discoloration, odour or gas formation observed during the 30 day storage period, except in the extract-lactose mixture where caking was evident at 40 degrees celcius. While discoloration can be seen with the extract-starch mixture at room temperature. At day 60, extract-talc and extract-starch mixtures showed discoloration at both storage temperatures while caking was

observed for extract-lactose mixture. Only the extract-MS mixture remained stable after 60 days of storage.

Discussion

This study for the first time reports the antidiabetic, antioxidant and hepatoprotective effects of the ethanolic extract of Diospyros pilosanthera, and its stability with excipients usually used to formulate a tablet dosage form. From the results, my study indicates that D. pilosanthera ethanolic extract possesses a good antidiabetic and antioxidant activities in STZ induced diabetic rats as well as hepatoprotective activity in DENA- induced liver toxicity. Furthermore, stability with excipients is time and temperature dependent.

Streptozocin selectively destroys the pancreatic insulin secreting β-cells, leaving less active cells and resulting in a diabetic state14. STZ-induced diabetes is characterized by severe loss in body weight15. In my study, hyperglycemia was observed after 3 days of STZ induction. Treatment with D. pilosanthera extract in STZ-induced diabetic rats, dose dependently normalized significantly the elevated blood sugar level after 15 days. The antidiabetic effect of the extract at 1000 mg/kg b.w. dose was found to be the most effective dose comparable to the reference drug Glibenclamide and the normal control. Same dose also showed the greatest effect in controlling the loss of body weight of diabetic rats.

Oxidative stress in diabetes has been shown to co-exist with a reduction in the endogenous antioxidant status16. Glutathione plays an important role in the endogenous non-enzymatic anti-oxidant system. Primarily it acts as a reducing agent and detoxifies hydrogen peroxide in the presence of an enzyme glutathione peroxidase17. The depleted glutathione may be due to reduction in GSH synthesis or degradation of GSH by oxidative stress in STZ-induced diabetic rats18. Treatment of D. pilosanthera extract significantly elevated the reduced glutathione levels towards normal in diabetic rats in a dose dependent manner.

Catalase is a free radical enzyme catalyzing the detoxification of hydrogen peroxide to water and oxygen19. It was reported that STZ-induced diabetes inhibits catalase activity20. Similar finding was observed in my study. Treatment of D. pilosanthera extract significantly recovered the CAT activity towards normal in a dose dependent manner.

AST, ALT and ALP activities in blood serum are generally accepted as an index of liver damage and this tendency is also known to be distinct in rodents21. Elevation of serum biomarker enzymes such as AST, ALT and ALP was observed in toxic livers of rats induced with DENA indicating impaired liver functions which may be due to hepatic damage. Fourteen days of treatment with D. pilosanthera extract restored all the serum biomarker enzymes to normal levels in a dose dependent manner.

Lipid peroxidation and associated membrane damage are key features of DENA-induced toxicity22. Free radical scavenging enzymes such as reduced glutathione and catalase provide the first defense against oxidative damage. Results show that administration of 1000 mg/kg b.w. dose of D. pilosanthera elevated the GSH levels towards normal and significantly increased CAT activities comparable to Sillimarin treated group while the DENA control group showed the opposite which could be due to over-utilization of these enzymatic antioxidants to scavenge the products of lipid peroxidation.

Histological observations clearly show that DENA in carcinogen control group animals clearly damages the normal architecture of hepatic tissue. A classic feature of DENA toxicity is centrilobular necrosis, dilated sinusoidal spaces and necrosis with blood pooling in sinusoidal spaces and central vein23. The liver tissue shows enlarged nuclei, disruption of the polyhedral shape of hepatocytes and abundant cytoplasm, indicative of cellular swelling. Pooling of red blood cells in the sinusoidal spaces is also observed along with cellular necrosis. Treatment with 1000 mg/kg b.w. dose of D. pilosanthera for 14 days resulted to a better liver architecture in DENA-induced hepatotoxic rats which is comparable with liver of Sillymarin-treated rats.

The possible mechanism of liver protection exhibited by D. pilosanthera extract was not studied but it can be assumed that the hepatoprotective effect is mediated through its antioxidant and/or free radical scavenging activity. Diospyros species were found to contain terpenoids, phenolic compounds, and flavonoids24 which is responsible for its antioxidant and antidiabetic activities. Literatures have

shown medicinal plants with hepatoprotective properties to mediate their protection due to high concentrations of flavonoids and alkaloids25. Phytochemical studies of D. pilosanthera exhibited the presence of tannins, phenols, alkaloids, triterpenes and flavonoids which may be responsible for its antidiabetic, antioxidant and hepatoprotective activities.

Assessment of possible incompatibilities between an active dug substance and excipients forms an important part of a preformulation stage during the development of a solid dosage form. Preformulation study of D. pilosanthera extract with the different excipients showed that degradation of the extract was time and temperature-dependent as shown from the results of the organoleptic examination.

CONCLUSION

It can be inferred from the data's presented that the administration of ethanolic extract of D. pilosanthera to STZ-induced diabetic rats demonstrated prominent reduction in blood sugar level, prevented weight loss, significantly elevated reduced glutathione levels and recovered catalase activity towards normal. Furthermore, treatment with D. pilosanthera extract to DENA-induced hepatotoxic rats restored levels of serum enzyme biomarkers to normal, enhancing activities of reduced glutathione and catalase, and maintained normal hepatic architecture. The mechanism of action for its antiidiabetic and hepatoprotective activities is yet to be investigated but may plausibly involve the endogenous antioxidant mechanisms of Rutin, tannins, phenols, alkaloids, triterpenes and flavonoids.

Furthermore, the stability of D. pilosanthera ethanolic extract is time and temperature dependent. Hence, it should be stored in a tightly sealed amber glass container at a temperature not higher than 30°C.

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