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ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITIES OF THE ETHANOLIC EXTRACT OF THE LEAVES OF DIOSPYROS PILOSANTHERA BLANCO (FAMILY EBENACEAE) IN DIETHYLNITROSOAMINE-INDUCED RAT LIVER TOXICITY

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ABSTRACT

Liver cancer is 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 antioxidant and anticancer properties. This study was carried out to investigate the antioxidant and hepatoprotective properties of *D. pilosanthera*, an endemic Philippine plant, in Diethylnitrosoamine-induced rat liver toxicity. 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 catalase, glutathione, alanine**a**minotranferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were evaluated to determine the antioxidant and hepatoprotective activities of *Diospyros pilosanthera*. Histopathological examination of the liver tissues of the rats induced with DENA was also done. 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 the studies indicate that the ethanolic extract exhibited significant antioxidant and hepatoprotective activities.

KEYWORDS: Hepatoprotective, carcinogenesis, phytomedicine, Diospyros pilosanthera.

INTRODUCTION

Hepatocarcinoma is the fifth most common cancer, and that majority of patients with this type of cancer will die within one year.[1,2a] 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] Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons that can be formed when oxygen interacts with certain molecules forming reactive oxygen species (ROS).[4]

Reactive oxygen species (ROS), when continuously produced in vivo, result in cell death and tissue damage.[5] Hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative damages.[6] 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. [7]

In spite of tremendous advances in modern medicine, there are not many effective drugs available that stimulate liver function, offer protection to the liver from damage or help to regenerate hepatic cells.[8] Lately, there is an increasing interest in natural antioxidants (e.g. polyphenols), present in medicinal and dietary plants, which might help prevent oxidative damage.[9] Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress.[10] Plants constitute a natural source of a diverse array of antioxidant molecules that have the potential to eliminate or neutralize the deleterious ROS.[11]

Diospyros species has been reported to have pharmacological applications arising from its extensive folkloric uses. The claims include antioxidant and antitumor properties. *Diospyros* species were proven to have good antioxidant activity. This includes: *D. lotus*,[12] *D.*

malabarica,[13] *D. preussi,*[14] *D. kaki,*[15] *D. discolor,*[16] D. *sanza-mimika,*[17] *D. peregrina,*[18] and *D. abyssinica.*[19] *Diospyros* is said to contain Diospyrin, a bisnaphthoquinone, which is present in the heartwood of many species which is well known for its anti-cancer activity.[20] In Taiwan, the hexane extract of the stem parts of *D. morrisiana* was found to show significant cytotoxicity against in vitro tissue culture cells of human KB and A-549 lung carcinoma, HCT-8 colon tumour and murine P-388 and L-1210 lymphocytic leukemia. *Diospyros lotus* extract was also found to have a high inhibitory activity against Lung large cell carcinoma COR-L23 and displayed a good antioxidant activity. [21] Apoptosis was shown to occur in HT-29 cells when administered with *D. kaki* extract. It also showed cytotoxicity against HeLa and PANC-1 cells as well as HT-29 cells. The results confirmed the potential use of *D. kaki* as a chemotherapeutic agent against human cancer cells. [22] *Diospyros montana* was found to contain Diospyrin which was proven to posses tumor inhibitory activity.[23] *Diospyros maritima* was found to be potently cytotoxic against cancer cell lines such as human oral epidermoid carcinoma (KB), human lung cancer (Lu1), hormone dependent human prostate cancer (LNCap) and human umbilical vein endothelial cells (HUVEC). [24] 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. Thus, this study was carried out to investigate the antioxidant and hepatoprotective properties of *D. pilosanthera,* an endemic Philippine plant.

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. Chemical used to induce liver toxicity like DENA, as well as reagents to test for catalase and reduced glutathione activities were procured from Sigma-Aldrich and Merck Incorporated. Positive control- sillymarin was bought from Sigma-Aldrich.. 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 were 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 21^0C-23^0C. 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 auto sampler. Separation of the compounds was effected through a C-18 reverse phase column of an Agilent Series II-HPLC machine at 30^0C. 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.[25] 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.[26]

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 (OECD, 2008). 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.

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 sillymarin (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. [2b]

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. [27]

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 37^{0}C and added with 25 µL of substrate. The absorbance was taken at 340 nm every minute for three minutes. [28a]

Aspartate Aminotransferase Assay

A standard kit was used. The kit is composed of a buffer containing L-aspartate (300mM), lactate dehydrogenase (1.13kU/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 37^0C 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. [28]

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 37^0C. 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. [28]

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 4^0C using a cooling centrifuge and the supernatant liquid were used. [2c]

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 H_2O_2 (19mM) in phosphate buffer, and 195 µL of Phosphate buffer (50mM, pH 7.4). The rate of H_2O_2 decomposition was measured at 240 nm per minute and catalase activity in U/l was calculated using the molar absorption coefficient of H_2O_2 (43.6 $M^{-1}cm^{-1}$) at 240 nm. [29, 30]

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 $M^{-1}cm^{-1}$ at 412 nm). [31]

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. [32] 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.

Statistical Analysis

The 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. [33]

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 2^0C-8^0C. 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 (LD_{50}) 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.[34] 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 of the plant has been reported by Rojo (1999) to be edible. 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.

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 Dragendorff's Valser's Hager's Wagner's	Yellow cream ppt red ppt w/ precipitation yellow ppt reddish-brown ppt	Yellow cream ppt (+++) Red-orange ppt (++) white ppt (+++) yellow ppt (+++)reddish-brown ppt(+++)	Presence of alkaloids
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

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*.

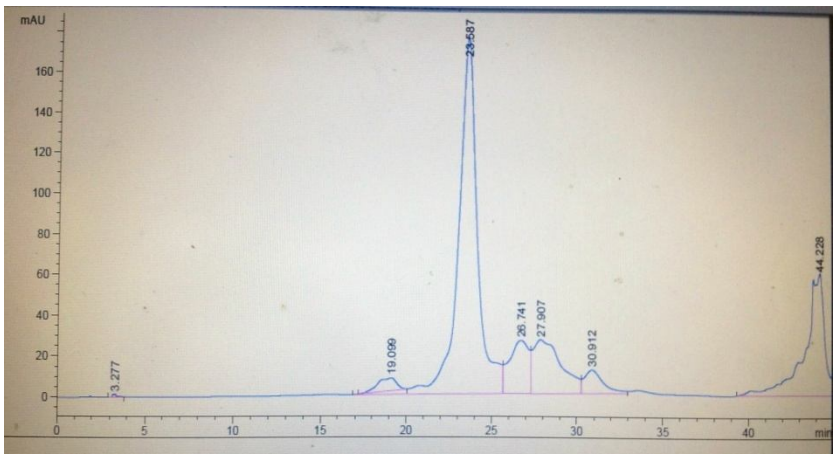

Figure 1. Chromatogram of D. Pilosanthera Extract at 365 Nm Showing

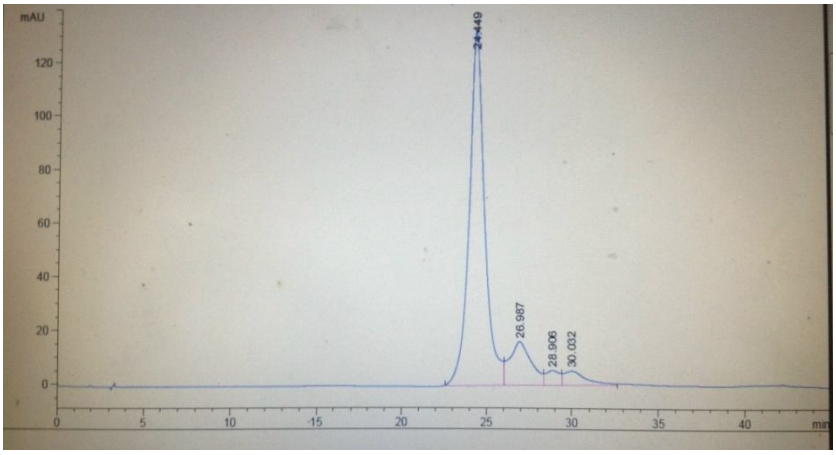

Figure 2. Chromatogram of Rutin at 365 Nm Showing Peak Retentiom Time At 24.449 Mins.

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 2, 3 and 4 show the summary of results.

Table 2. 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]; posthoc: [(A=E=F)<D< C<B]

Table 3.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 4. 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	119.97 ± 6.39	171.84 ± 4.76	10.333	<0.001

extract w/ DENA				
[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]

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 5 and 6. 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.

Table 5. 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 6: 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

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.

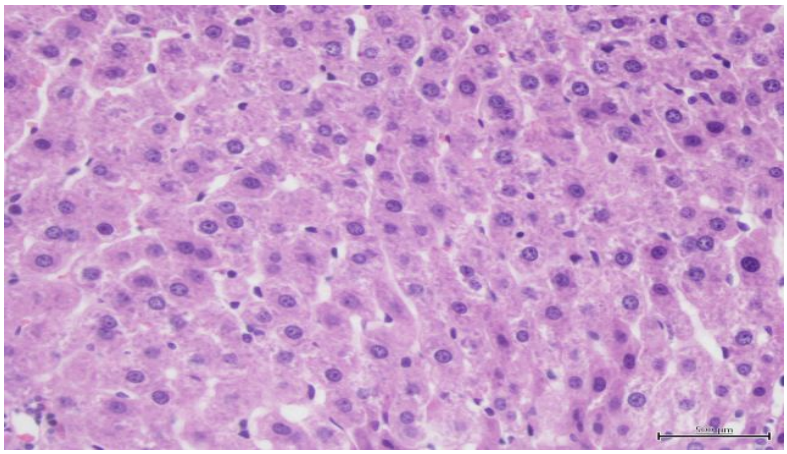

Figure 3. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the control group. Liver shows intact central vein surrounded by healthy hepatocytes with well-preserved cytoplasm and prominent nucleus and nucleolus, and sinusoids between hepatocytes.

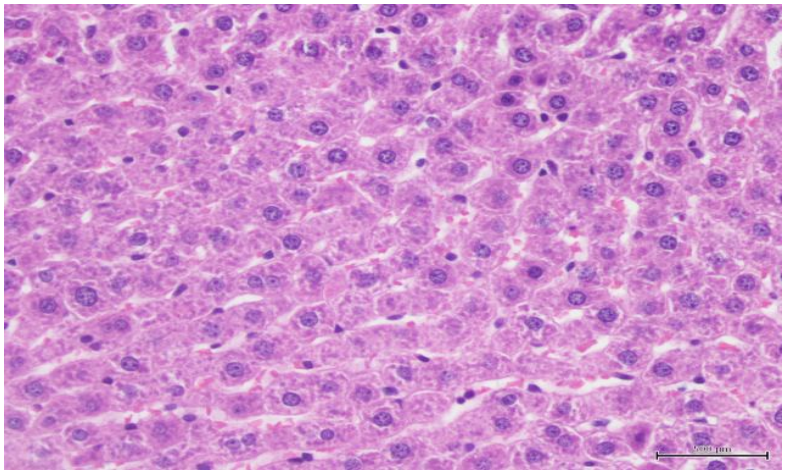

Figure 4. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the DENA toxicant group. Liver tissue reveals centrilobular necrosis, with blood pooling in sinusoidal space.

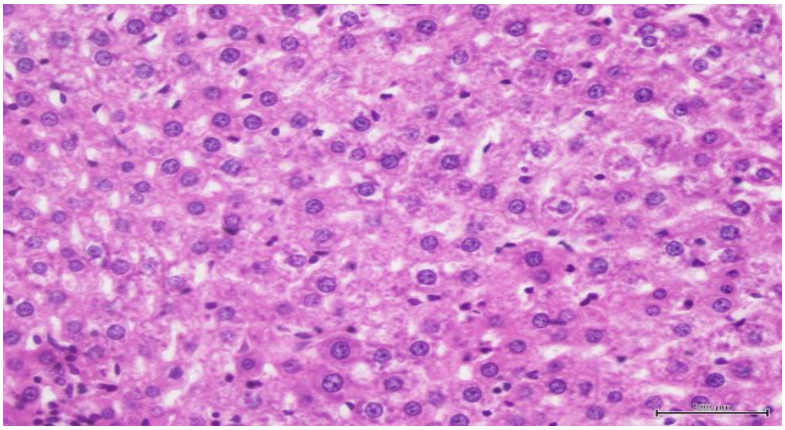

Figure 5. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the 200 mg/kg-dose extract treated group. Image shows cellular swelling and moderate scattered inflammatory cell infiltrates consisting of neutrophils, lymphocytes and Kupffer cells.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

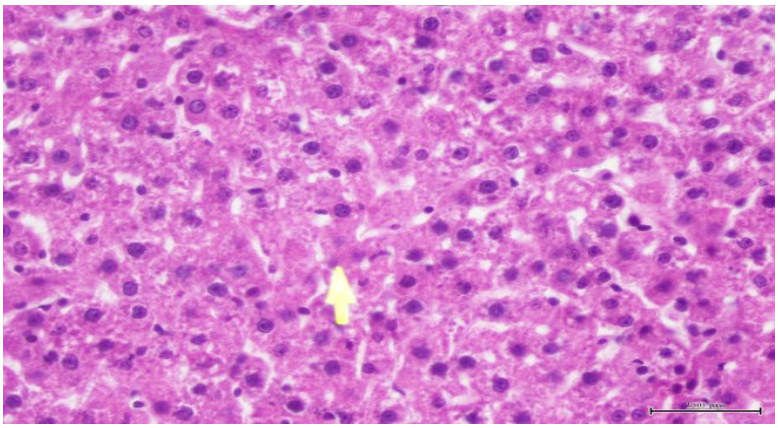

Figure 6. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the 500 mg/kg-dose extract treated group. Image shows scant necrosis (as pointed by the yellow arrow), cellular swelling and fewer scattered inflammatorycell.

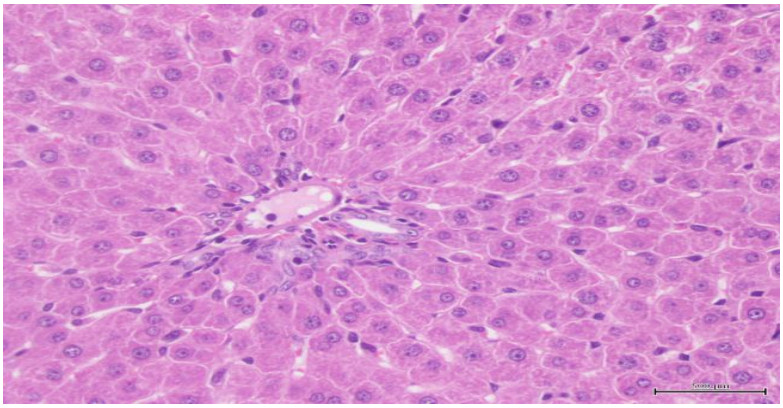

Figure 7. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the 1000 mg/kg-dose extract treated group. Image shows a central vein surrounded by normal hepatocytes. Regenerative changes are present as seen from the binucleated cells. Kupffer cells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

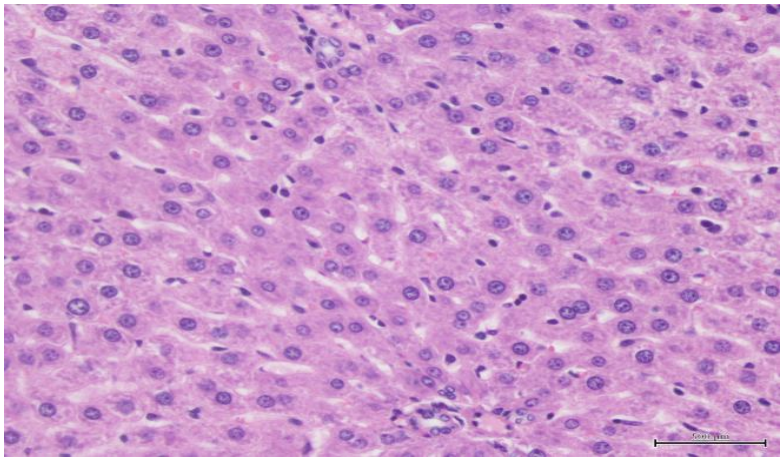

Figure 8. Light Microphotograph (400X) of hematoxylin and eosin-stained section of formalin-fixed hepatic tissue from the Sillymarin-dose extract treated group. A normal centrilobular vein is shown surrounded by normal hepatocytes. Regenerative changes are evident as shown by the binucleated cells. Kupffer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

DISCUSSION

Currently, liver diseases remain as one of the serious health problems. However, we do not have satisfactory liver protective drugs in allopathic medical practice for serious liver disorder.[2d] Herbal drugs play a role in the management of liver disorders. [35]

This study for the first time reports the antioxidant and hepatoprotective effects of the ethanolic extract of *Diospyros pilosanthera*. From the results, my study indicates that *D. pilosanthera* ethanolic extract possesses a good antioxidant and hepatoprotective activity in DENA- induced liver toxicity.

During carcinogenesis, some enzymes can be used as an biochemical indicators of tumor response to therapy.[36] 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 rodents.[37] 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 toxicity. [38,2e] Lipid peroxidation plays an important role in carcinogenesis (Banakar, et al., 2004). 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.[39] Aclassic feature of DENA toxicity is centrilobular necrosis, dilated sinusoidal spaces and necrosis with blood pooling in sinusoidal spaces and central vein.[40] 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 flavonoids.[41] which is responsible for its antioxidant and antidiabetic activities. Terpenoids were reported to have free radical scavenging activity and antioxidant capacity in diabetes.[42,43] Literatures have shown medicinal plants with hepatoprotective properties to mediate their protection due to high concentrations of flavonoids and alkaloids.[44,45] *Phytochemical studies of D. pilosanthera exhibited the presence of* tannins, phenols, alkaloids, triterpenes and flavonoids which may be responsible for its antioxidant and hepatoprotective activities.

CONCLUSION

It can be inferred from the data's presented that the administration of ethanolic extract of *D. pilosanthera* to DENA-induced liver 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 antioxidant and hepatoprotective activities is yet to be investigated but may plausibly involve the endogenous antioxidant mechanisms of Rutin, tannins, phenols, alkaloids, triterpenes and flavonoids.

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REFERENCES

1. Shaarawy, S.M., Tohamy, A.A., Elgendy, S.M., Elmageed, Z.Y.A., Bahnasy, A., Mohamed, M.S., Kandil, E., and Matrougui, K. Protective Effects of Garlic and Silymarin on NDEA-induced Rat Hepatoxicity. International Journal of Biological Sciences, 2009;5(6): 549-557
2. Raghuveer, C., and Tandon, R.V. Consumption of Functional Food and Our Health Concerns. Journal of Physiology, 2009;5(1): 76-83
3. Pracheta, Sharma, V., Paliwal, R., Sharma, S., Singh, L., Janmeda, B.S., Savita, Yadav, S., &and Sharma, S.H. Chemoprotective Activity of Hydroethanolic Extract of *Euphorbia nerifolia* Linn Leaves Against DENA-induced Liver Carcinogenesis in Mice,Biology and Medicine, 2011;3(2): 36-44
4. Haliwell, B. Free Radicals, Antioxidants, and Human Disease Curiosity, Cause, Or Consequence? The Lancet, 1994; 344(8924): 721-724
5. Rao, K.S., Chaudhury, P.K. and Pradhan, A. Evaluation of Anti-oxidant ACtivites and Total Phenolic Content of C*hromolaena odorata*. Food and Chemical Toxicology, 2009;48: 729-732
6. Ilango, K., and Chitra, V. Hepatoprotective and Anti-oxidant Activities of Fruit Pulp of *Limonia acidissima* Linn. International Journal of Health Research, 2009;2(4): 361-367
7. Rahimi, R., Nikfar, S., Larijani, B. And Abdollahi, M. A Review on the Role of Antioxidants in the Management of Diabetes and its Complications. Biomedicine and Pharmacotherapy, 2005; 59: 365-373
8. Chattopadhyay, M.B., Mukherjee, S. Kulkarni, I., Vijayan, V., Doloi, M., Kanjilal, N.B. and Chatterjee, M. Proton-Induced X-Ray Emission (PIXE) Analysis And DNA-Chain Break Study In Rat Hepatocarcinogenesis: A Possible Chemopreventive Role By Combined Supplementation Of Vanadium And Beta-Carotene. Cancer Cell International, *2005;* 5:16.

9. Silva, B.A., Ferreres, F., Malva J.O., and Dias, A.C.P. Phytochemical and Antioxidant Characterization of *Hypericum perforatum* Alcoholic Extracts. Food Chemistry, 2005;90:157-167
10. Ozsoy, N., Can, A., Yanardag, R. and Akev, N. Antioxidant Activity of *Smilax excels* Leaf Extracts. Food Chemistry, 2008;110:571-583
11. Shahidi, F. and Naczk, M. Phenolics in Food and Nutraceuticals. CRC Press. Boca Raton. Florida: 2004
12. Loizzo, M.R., Said, A., Tundis, R., Hawas, U.W., Rashed, K., Menichini, F., Frega, N.G. and Menichini, F. Antioxidant and Antiproliferative Activity of D. lotus L. extract and isolated Compounds. Plant Foods Human Nutrition, 2009;64:264-270
13. Mondal, S.K., Chakraborty, G., Gupta M. and Mazumder, U.K. In vitro Activity of *Diopsyros malabarica* Kostel Bark. Indian Journal of Experimental Biology, 2006;44:39-44
14. Okonkwo, T.JN. and Okonkwo, C.JO. (2009). Antioxidant Properties of *Diospyros preussi* (Ebenaceae Gurke) Seed Oil. Tropical Journal of Pharmaceutical Research, 2009; 8(6):551-555
15. Jung, S.T., Park, Y.S., Zachwieja, Z., Folta, M., Barton,H., Piotrowicz, J., katrich,E., Trakhtenberg, S. and Gorinstein, S. Some Essential Phytochemicals and the Antioxidant Potential in Fresh and Dried Persimmon. International Journal of Food Sciences and Nutrition,2005;56(2):105-113
16. Lee, E.O., Lee, J.R., Kim, K.H., Baek, N.I., Lee, S.J., Lee, B.H., Cho, K.D., Ahn, K.S., and Kim, S.H. The methylene chloride fraction of trichosanthis fructus induces apoptosis in U937 cells through the mitochondrial pathway. Biol. Pharm. Bull., 2006; 29: 21-25.
17. Tangmouo, J.G., Ho, R., Lannang, A.M., Komguem, J., Lontsi, A.T., Lontsi, D. and Hostettmann, K. Norbergenin Derivatives from the Stem Bark of D. sanza-minika (Ebenaceae) and their Radical Scavenging Activity. Phytoschemistry Letter, 2009; 2:192-195
18. Dewanjee, S., Das, A.K., Sahu, R. and Gangopadhyah, M. Antidiabetic Activity of Diospyros peregrine fruit: Effect of Hyperglycemia, Lyperlipidemia and Augmented Oxidative Stress in Experimental Type 2 Diabetes. Food Chemistry Toxicology, 2009; 47: 2679-2685
19. Maiga A, Malterud KE, Diallo D, Paulsen BS Antioxidant and 15- Lioxygenase inhibitory activities of the Malian medicinal plants *Diospyros abyssinica* (Hiern) F. White

(Ebenaceae), *Lannea velutina* A. Rich (Anacardiaceae) and *Crossopteryx febrifuga* (Afzel) Benth. (Rubiaceae). J. Ethnopharmacol., 2006; 104: 132-137

20. Sagar, S., Kaur, M., Minneman, K. and Bajic, V. Anticancer Activities of Diospyrin, its Derivatives and Analogues. European Journal of Medicinal Chemistry, 2010; 45:3519-3530.
21. Jo, K.J., Lee, J.M., Lee, S.C. and Park, H.R. Anticancer Activity of Persimmon *(Diospyros kaki* L.) Calyx Extracts on Human Cancer Lines; Journal of Medicinal Plants Research, 2011; 5(12):2546-2550
22. Loizzo, M.R., Said, A., Tundis, R., Hawas, U.W., Rashed, K., Menichini, F., Frega, N.G. and Menichini, F. Antioxidant and Antiproliferative Activity of D. lotus L. extract and isolated Compounds. Plant Foods Human Nutrition, 2009; 64:264-270
23. Ravishankara, M.N., Shrivastava, N., Jayathirtha, M.G., Padh, H. and Rajani, M.Sensitive High performance Thin Layer Chromatographic Method for the Estimation of \Diospyrin, a Tumour Inhibitory Agent from the Stem bark of *D. montana* Roxb. Journal of Chromatography, 2000; 744:257-262
24. Gu, J.Q., Graf, T.N., Lee D., Chai, H.B., Mi, Q., Kardono, L.B.S., Setyowati, F.M., Ismail, R., Riswan, S., Farnsworth, N.R., Cordell, G.A., Pezzuto, J.M., Swanson, S.M., Kroll, D.J., Falkinham III, J.O., Wall, M.E., Wani, M.C., Kinghorn, A.D. and Oberlies, N. H. Cytotoxic and Antimicrobial Constituents of the bark of *Diospyros maritima* Collected in two Geographical Locations in Indonesis. Journal of Natural Products, 2004; 67:1156-1161
25. Dharmender, R., Deepti, R., Sushila, R. and Drapete, K. HPTLC densitometric quantification of sigmasterol and lupeol from Fiscus religiosa. Arabian Journal of Chemistry, 2011; 10(106):10-21
26. Liu, Y.T., Lu, B.N. and Peng, J.Y. Hepatoprotective activity of the total flavonoids from *Rosa laevigata* fruit in mice treated by paracetamol. Food Chemistry, 2011; 125: 719-725
27. Shyam Kumar, B., Gnanasekaran, D., Jaishree, V., and Channabasavaraj, K.P. "Hepatoprotective activity of *Coccinia indica leaves extract", Int J Pharm Biomed Res,* 2010; 1(4), 154-156
28. Human Diagnostics. GPT (ALAT) IFCC Mod. LiquiUV Test Alanine Aminiotransferase. Retrieved from March 31, 2013, Standard Chemical and Pharmaceutical CompanyWebsite,2011.http//www.standard.com.tw/standard/system_manager/tw/products/uploadFile/49/En-gptli%28GPT%29.pdf

29. Aebi, H.E. and Bergmeyer, H.O. (eds.). Catalase, methods enzzymology. (p.2). New York: Academic Press: 1983; 2.
30. Bogdanska, J.J., Kornetti, P. and Todorova, B. Erythrocyte superoxide dismutase, glutathione peroxidase and catalase activites in healthy male subjects in Republic of Macedonia. *Bratisl Lek Listy*, 2003;104: 108-114
31. Saeed, N., Khan, M.R. and Shabbir, M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts Torilis leptophylla L. Biomed Central Complementary and Alternative Medicine, 2012; 12:1-12
32. Yousef, M.I., Omaar, S.A.M., El-Guendi, M.I. and Abdelmegid, L. A. Potential protective effects of quercetin and curcumin on paracetamol-induced histological changes, oxidative stress impaired liver and kidney functions and haemototoxicity in rat. Food and Chemical Toxicology, 2010; 48: 3426-3451
33. Duncan, B.D. Multiple Range Test for Correlated and Heteroscedastic Means. Biometrics,1957; 13:359-364
34. Organization for Economic Cooperation and Development. Test No. 425: Acute Oral Toxicity: Up and Down Procedure. OECD Guidelines for the testing of chemical, Section 4 Helath Effects: 2008; 10: 1787
35. Subramoniam A, Evans DA, Rajasakhran SP. Hepatoprotective activity of *Trichopuszeyl anicus* extracts against paracetamol induced damage in rats. Ind J Expt Biol, 1998; 36: 385-389.
36. Thirunavukkarasu, S., and Sakthisekaran, D. Effect of Selenium on NEDA-induced Multistage Hepatocarcinogenesis with Reference to Lipid Peroxidation and Enzyme Antioxidants. Cell Biochemistry and Function, 2003; 19:27-35
37. Ha, W.S., Kim, C.K., Sung, S.H., and Kang, C.B. Study on the mechanism of multistep hepatotumorigenesis in rat: development of hepatotumorigenesis. J Vet Sci, 2001; 2: 53–58.
38. Anis, K, V., Rajesh, K.N.V., and Kuttan, R. Inhibition of Chemical Carcinogenesis by Berberine in Rats and Mice. Journal of Pharmacy and Pharmacology, 2001;53:763-768
39. Chattopadhyay, M.B., Mukherjee, S. Kulkarni, I., Vijayan, V., Doloi, M., Kanjilal, N.B. and Chatterjee, M. Proton-Induced X-Ray Emission (PIXE) Analysis And DNA-Chain Break Study In Rat Hepatocarcinogenesis: A Possible Chemopreventive Role By Combined Supplementation Of Vanadium And Beta-Carotene. Cancer Cell International, 2005; 5:16

40. Sobiya Raj, D., jannet Vennila, J., Aiyavu, C. and Panneerselvam, K. The hepatoprotective effect of the alcoholic extract of Annona squamosa leaves on experimentally induced liver injury in swiss albino rats. International Journal of Integrative Biology, 2009; (5)3: 181-186
41. Sagar, S., Kaur, M., Minneman, K. and Bajic, V. Anticancer Activities of Diospyrin, its Derivatives and Analogues. European Journal of Medicinal Chemistry, 2010; 45:3519-3530.
42. Jang, I.C., Jo, E.K., Bae, M.S., Lee, H.J., Jeon, G.I., Park, E., Yuk, H.G., Ahn, G.H. and Lee, S.C. Antioxidant and antigenotoxic activities of different parts of persimmon (Diospyros kaki cv. Fuyu) fruit. Journal of Medicinal Plants Research,2010;4(2):155-160.
43. Yasuda, K., Kizu, H., Yamashita, T., Kameda, Y., Kato, A., Nash, R.J., Fleet, G.W., Molyneux, R.J., and Asano, N. New Suga-mimic Alkaloids from Pods of Angylocalyx pynaertii. Journal Natural Products; 65(2): 192-202
44. Miller, N.J. and C.A. Rice-Evans. Factors influencing the antioxidant activity determined by the ABTS radical cation assay. Free Radic. Res. 26:195-199
45. Adeneye, A.A. and Agbaje, E.O. Pharmacological evaluation of oral hypoglycemic and antidiabetic effects of Morinda lucida Benth. Fresh leaves ethanol extract in normal and alloxan-induced diabetic rats. African Journal of Biomedical Research, 2008; 11(1), 65-71.