Hepatoprotective Effect of Methanol Extract of *Diospyroschloroxylon* Leaf in *N*-Nitrosodimethylamine-induced hepatotoxicity in rats

G.E.Adeleke[1, 2], O.T.Adedosu[1], O.A.Adaramoye[2], A.S.Olagunju[1], O.J.Teibo[1], O. O.Arinde[1], O. K. Afolabi*[1]

[1]Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria
[2]Department of Biochemistry, University of Ibadan, Oyo State, Nigeria

ABSTRACT

The present study investigated the ameliorative properties of *Diospyroschloroxylon* (methanolic) leaf extract (DCLE) in liver of rats intoxicated with N-Nitrosodimethylamine (NDMA). Antioxidant markers- Superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and lipid peroxidation were assayed spectrophotometrically. DNA fragmentation was determined spectrophotometrically. The Bcl-2, Ki-67 and CD15 markers were assayed using immunohistochemistry. The activities of SOD and catalase were decreased in the NDMA-treated rats by 99.5% and 47.1%, respectively compared with controls, whereas pretreatment with DCLE showed significantly elevated ($p < 0.05$) values as against NDMA. NDMA treatment reduced GSH level by 35.7% compared with controls, while DCLE pretreatment significantly increased the level relative to NDMA group. The NDMA significantly increased ($p < 0.05$) MDA level with the value 3.56 ± 1.07 (µM of MDA/mg protein) as against the control (1.83 ± 0.14 µM of MDA/mg protein). On pretreatment with DCLE, the level was observed to be 1.99 ± 0.77 µM of MDA/mg protein. NDMA treatment significantly increased DNA fragmentation in the liver by 122.2% relative to control rats, while DCLE pretreatment reduced the level by 77.9% compared with NDMA treatment. The NDMA caused strong expressions of Bcl-2, Ki-67 and CD15 relative to controls, while DCLE supplementation ameliorated the effects. The findings suggest that Diospyroschloroxylon methanol leaf extract possesses hepatoprotective properties expressed through the amelioration of oxidative stress, cell proliferation and malignancy in liver of rats treated with *N*-Nitrosodimethylamine.

Key words: N-Nitrosodimethylamine, Diospyroschloroxylon, Antioxidant indices, DNA fragmentation, Cell proliferation

Introduction

N-Nitrosodimethylamine (NDMA) is a potent toxic compound unintentionally formed during various industrial manufacturing processes, from where it becomes introduced into the environment [1]. Studies have established the presence of this compound in foods, drinking water, ground water and wastewater [2]. *N*-Nitrosamines are mutagenic and are capable of generating deleterious radicals in cellular systems [3]. However, these compounds can also be synthesized in man as a result of nitrosation of nitrosable precursors in diets, drinking water, drugs and tobacco smoke [4, 5]. In mammalian cells, NDMA is metabolically demethylated via a cytochrome P-450-dependent initial α-hydroxylation reaction to form methyldiazohydroxide[6, 7]. The methyldiazohydroxide is finally converted to methyldiazonium ion, which can methylate DNA and other macromolecules to initiate carcinogenesis [8]. It has been shown that the metabolism of *N*- nitrosamines could generate a high level of nitric oxide [9]. Earlier studies have revealed the generation of reactive oxygen species (ROS) during cellular metabolism of *N*-nitrosamines which may lead to formation of reactive oxygen species (ROS), which could trigger tumor development [10, 11] and immunoregulation[12].*Diospyros*is a large genus of trees or shrubs, belonging to family Ebenaceae, which are widely distributed all over the world. Phytoconstituents of *Diospyros*range from hydrocarbons, long chain fatty acids to steroids, terpenoids, naphthoquinones, and naphthalene-based aromatics and aurones. The leaves are used in curing several diseases including boils, pains, swelling and skin diseases, to mention a few [13]. However, the fruits of some *Diospyros* species contain some chemicals useful as fish poisoning or for medicinal purposes, although the

**Correspondence*
Dr. O. K. Afolabi
Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo State, Nigeria

active components are yet to be identified [14]. Earlier phytochemical studies of leaf extracts of *Diospyroschloroxylon* indicated the presence of a broad spectrum of secondary metabolites, such as cardiac glycosides, alkaloids, flavonoids, tannins, amino acids and proteins and saponins[15]. In a phytochemical study, it was reported that acetone extract of *D. chloroxylon* contained secondary metabolites such as terpenoids, alkaloids, tannins, phenols and saponins, while the aqueous and ethanol extracts were found to contain quinones, amino acids and proteins [16]. An important triterpene, known as betulinic acid, has been reported to have several biological properties, and is present in *Diospyrosleucomelas*[17]. The presence of these secondary metabolites in plants is an indication that plants are potential sources of useful phytomedicines[18]. The medicinal uses and chemical composition of various *Diospyros* species have been reviewed [19], and several metabolites in *Diospyros* species like ursolic acid have been found to exhibit an inhibitory potential against HIV-1 protease, while others, such as diospyrin, amytrin and betulin were found to be cytotoxic against many cancers [20]. Information on the activity of *Diospyroschloroxylon* against *N*-Nitrosodimethylamine intoxication was scarce in literature, the present study was therefore designed to investigate the activities of *Diospyroschloroxylon* in rats treated with intraperitoneal administration of NDMA.

Materials and method

Chemicals

N-Nitrosodimethylamine and methanol were purchased from Sigma Aldrich (St. Loius, MO, USA). All other chemicals and reagents used in this study were commercially available and are of analytical grade.

Plant material

The leaves of*Diospyroschloroxylon*purchased from a local herb vendor in Ogbomoso, Nigeria, were authenticated at the Department of Biology, Botany Unit, Ladoke Akintola University of Technology, Ogbomoso, and a voucher specimen was deposited in the herbarium.

Extraction

The collected leaves were rinsed with distilled water, air dried and powered using an electrical blender. The powered leaf was soaked in methanol and allowed to stand for 72 hours. This process of extraction was repeated twice and the extract was collected, filtered and concentrated under vacuum using rotary evaporator at 45°C. The crude extract obtained was used for the study.

Experimental Design

Twenty four male Wistar rats of an average weight of 140 ± 26 g were purchased from the Institute of Advanced Medical Research and Training (IAMRAT) Animal House, University College Hospital (UCH) Ibadan, Oyo State, Nigeria. The animals were randomly divided into four groups having six rats per group and then housed in cages and fed *ad libitum* with rat pellets and distilled water under 12- hours light/dark cycle at room temperature. The rats were acclimatized for one week. Control group was given normal rat pellets and water. The DCLE group was given daily oral intubation of *Diospyroschloroxylon* (methanolic) leaf extract (DCLE) (100 mg/kg) for 14 days. The N-Nitrosodimethylamine (NDMA) group was treated with intraperitoneal injection of NDMA at 5 mg/kg ($^{1}/_{5}{}^{th}$ LD_{50}) twice per week, while NDMA + DCLE group was administered with daily oral intubation of DCLE (100 mg/kg) for 14 days and intraperitoneal injection of NDMA (5 mg/kg) twice per week. After 14 days of treatment, rats were fasted overnight and weighed. Blood was collected by ocular bleeding and rats were sacrificed by cervical dislocation.

Collection of blood and liver

Blood was collected by ocular bleeding into test tubes, allowed to coagulate, and supernatant was centrifuged at 3000rpm for 10 minutes to obtain the serum which was kept under refrigeration (4°C) for biochemical analysis. Liver was excised, washed in 1.15% KCl solution (washing buffer) to remove blood and then weighed. The organ was divided into two portions. One portion was preserved in 10% formalin for immunohistochemical study, while the other portion was homogenized with Teflon homogenizer in 0.01M Phosphate buffer solution (pH 7.4). It was then centrifuged at 3000rpm for 10minutes to obtain supernatant which was used for the determination of biochemical parameters.

Biochemical assays

Total protein assay

Total protein concentrations of serum and liver were determined using Lowry's method [21].

Assay of Superoxide dismutase (SOD) activity

Superoxide dismutase activity in liver homogenate was determined by the epinephrine method, measuring the absorbance at 480nm as previously described [22].

Assay of Catalase (CAT) activity

Catalase activity in liver homogenate was spectrophotometrically determined, measuring the absorbance at 240nm according[23].

Assay of Malondialdehyde (MDA) level

Lipid peroxidation in liver homogenate was estimated by determining the concentration of malondialdehyde (MDA). The measurement of MDA involved taking the absorbance of Thiobarbituric acid-reacting substances (TBARS) at 532nm [24]. The MDA concentration was calculated using a molar extinction coefficient (Ɛ) of 1.56×10^{5} $M^{-1}cm^{-1}$.

Assay of Reduced Glutathione (GSH)

Reduced Glutathione level in liver homogenate was determined, measuring the absorbance at 412nm as previously described [25].

DNA Fragmentation by Diphenylamine (DPA) Assay

The colorimetric determination of fragmented DNA was done according to the method described by Wu *et al*. [26] with some modifications. The liver was homogenized in Tris-EDTA (TE) buffer and then centrifuged at 27000 x g for 10 minutes, to separate the intact DNA (pellet) from the fragmented DNA (supernatant). Both the pellet and supernatant were treated with freshly prepared DPA reagent for colour development. Incubation was carried out at 37^{0}c for 16-24 hours. The absorbance was read spectrophotometrically at 620nm. The percentage fragmented DNA was calculated using the formula:

Fragmented DNA= Absorbance of supernatant / Absorbance of pellet + Absorbance of supernatant

Immunohistochemical (IHC) assays

Immunochemical studies of liver sections for expressions of Bcl-2, Ki-67 and CD15 were carried out according to Chakravarthi*et al*. [27] with modifications as briefly described below. The deparaffinized liver sections were subjected to the peroxidase labeled streptavidin-biotin technique, using the monoclonal antibodies against Bcl-2, Ki-67 and CD15 antigens. Peroxidase blocking was carried out on the sections by covering the sections with 3% hydrogen peroxide for 15 minutes. The sections were washed with phosphate buffered saline (PBS) and protein blocking was done using avidin for 15 minutes. The sections were washed with PBS and endogenous biotin in the liver was blocked for 15 minutes, followed by incubation with 5µg/ml each of anti-Bcl-2, anti-Ki-67 and anti-CD15 antibodies (diluted in 1:100) for 60 minutes. PBS was used to wash excess antibodies followed by application of secondary antibodies (LINK) on the sections for 15 minutes. The sections were washed and Horseradish peroxidase (HRP) label was applied on the sections for 15 minutes, and then washed with PBS for 5 minutes to remove unbound HRP. Visualization of the reaction products was done by immersing the section in Karnovsky solution (0.01% diaminobenzidine in Tris buffer containing 0.05% hydrogen peroxide and 0.01% sodium azide). Excess diaminobezidine (DAB) solution and precipitate were washed off with distilled water. The tissue sections were counterstained with Haematoxylin solution for 2 minutes. The slides were dehydrated in alcohol, cleaned in xylene, mounted in DPX mountant and then observed under microscope.Cells with specific brown colour in the cytoplasm, cell membrane or nuclei, depending on the antigenic sites, were considered positive.

Statistical Analysis

Data are expressed in Mean ± SD. Student T-test analysis was used for statistical comparison and differences were taken as significant at $p < 0.05$. Descriptive and graphical methods were used to characterize the data.

Results

The data in Table 1 indicate that NDMA increased weight gain in the rats by 22.8% when compared with the control rats. However, a combination of DCLE with NDMA showed that the weight gain was lowered (86.7%) relative to the NDMA-treated rats. The treatment showed no significant differences in the weight and relative weight of liver of the rats.

Table 1: Effects of DCLE on body weight and relative weights of liver of rats treated with NDMA

Treatments	Weight of rats (g)			Weight of liver (g)	Relative weight of liver (%)
	Initial	Final	Weight Gain		
Control	138.70 ±22.4	170.92 ±11.60	32.22 ±1.42	4.47 ± 0.64	2.62 ± 0.14
DCLE	141.34 ±40.2	172.00 ± 6.47	30.66 ± 3.13	5.31± 0.50	3.08 ± 0.18
NMDA	140.17±19.27	179.72± 19.80	39.55 ±1.78*	5.23 ± 0.45	2.91 ± 0.45
DCLE + NMDA	143.40±24.50	164.59± 12.89	21.19 ± 2.98**	5.54 ± 0.23	3.36 ± 0.14

Data are expressed as means ± SD (n= 5). Value with * is significantly different (p< 0.05) when compared with control while ** signifies significant difference (p< 0.05) compared with NDMA-group.
DCLE – *Diospyroschloroxylon* leaf extract, NDMA- *N*-Nitrosodimethylamine

Table 2 shows the effect of the different treatments on protein concentrations in the serum and liver of the rats. NDMA treatment was observed to significantly increase ($p < 0.05$) the serum and liver protein levels compared with controls, while pre-treatment with DCLE lowered the levels by 99.1% and 48.7%, respectively, compared with NDMA group.

Table 2: Effect of DCLE on total protein concentrations of serum and liver in rats treated with NDMA

Treatments	Serum (µmol/dL)	Liver (µmol/dL)
Control	29.51 ± 1.17	33.60 ± 4.17
DCLE	32.01± 7.28	24.09 ± 1.66
NDMA	39.34 ± 4.30*	51.30 ± 1.83*
DCLE + NMDA	19.76 ± 4.14**	34.49 ± 8.10**

Data are expressed as means ± SD (n= 5). Value with * is significantly different ($p< 0.05$) when compared with control while ** signifies significant difference ($p< 0.05$) compared with NDMA-group.
DCLE – *Diospyroschloroxylon* leaf extract, NDMA- *N*-Nitrosodimethylamine;. As shown in Table 3, the activities of both superoxide dismutase (SOD) and catalase (CAT) were decreased in the NDMA-treated rats by 99.5% and 47.1%, respectively in liver, whereas pretreatment with DCLE elevated the activities by 59% and 89.7%, respectively in comparison with the rats administered with NDMA. The level of (GSH) in the hepatic tissue was noticed to significant decrease ($p < 0.05$) in the NDMA group compared with controls. On pretreatment with DCLE, the level was found to increase by 51.5% when compared with the NDMA-treated rats (Table 3). As also shown in table 3, NDMA significantly increased ($p < 0.05$) the level of lipid peroxidation measured as malondialdehyde (MDA) in the rats with the value 3.56 ± 1.07(µM of MDA/mg protein) as against the control (1.83 ± 0.14 µM of MDA/mg protein). On pretreatment with DCLE, the level was observed to be 1.99 ± 0.77 µM of MDA/mg protein. This result shows that NDMA increased the MDA level by 94.5% compared with controls, while pretreatment with DCLE reduced the level by 78.9%, relative to NDMA-treated rats.

Table 3: Effects of DCLE on antioxidant indices in liver of rats treated with NDMA

Treatments	SOD (U/mg protein)	CAT (U/mg protein	GSH (U/mg protein)	MDA (µM of MDA/mg protein
Control	7.50 ± 0.45	6.25 ± 1.33	13.75 ± 2.11	1.83 ± 0.14
DCLE	9.23 ± 1.12	5.45 ± 0.22	11.67 ± 0..98	2.15 ± 0.76
NDMA	3.76 ± 0.19*	4.25 ± 1.00*	10.13 ± 1.21*	3.56 ± 1.07*
DCLE + NDMA	5.98 ± 0.23**	8.06 ± 2.65**	15.35 ± 1.88**	1.99 ± 0.77**

Data are expressed as means ± SD (n= 5). Value with * is significantly different ($p< 0.05$) when compared with control while ** signifies significant difference ($p< 0.05$) compared with NDMA-group.
DCLE – *Diospyroschloroxylon* leaf extract, NDMA- *N*-Nitrosodimethylamine
Figure 1 depicts the effect of DCLE on DNA fragmentation in liver of rats treated with NDMA.DNA fragmentation in the hepatic tissue was significantly increased by NDMA (90.43 ± 1.71 %) compared with controls (40.70 ± 1.15 %). On pretreatment with DCLE, it was found that DNA fragmentation decreased significantly (50.83 ± 3.11%) against NDMA effect in the experimental rats. This result has therefore revealed that NDMA treatment significantly increased DNA fragmentation in the liver by 122.2 % relative to control rats, while DCLE pretreatment reduced the level by 77.9% compared with NDMA treatment.

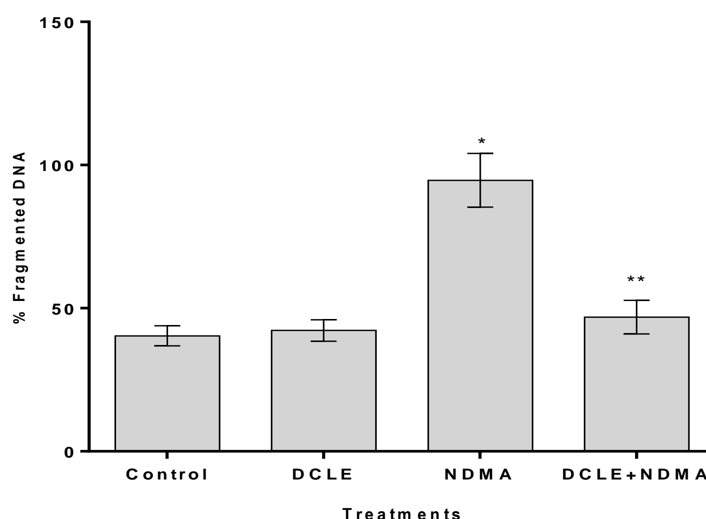

Figure 1: Effects of DCLE on DNA fragmentation in liver of rats treated with NDMA

Data are expressed as means ± SD (n= 5). Value with * is significantly different ($p< 0.05$) when compared with control while ** signifies significant difference ($p< 0.05$) compared with NDMA-group.

DCLE – *Diospyroschloroxylon* leaf extract, NDMA- *N*-Nitrosodimethylamine

Immunohistochemical examination results for Bcl-2, Ki-67 and CD15 expressions in liver are shown in figures 2, 3 and 4, respectively. Bcl-2 was expressed at a moderate level in both the control and DCLE groups (Fig. 2A and 2B, respectively), while NDMA treatment resulted in a pronounced expression of the same (Fig. 2C). This was however, attenuated in the NDMA + DCLE group by pre-treatment with DCLE (Fig. 2D).

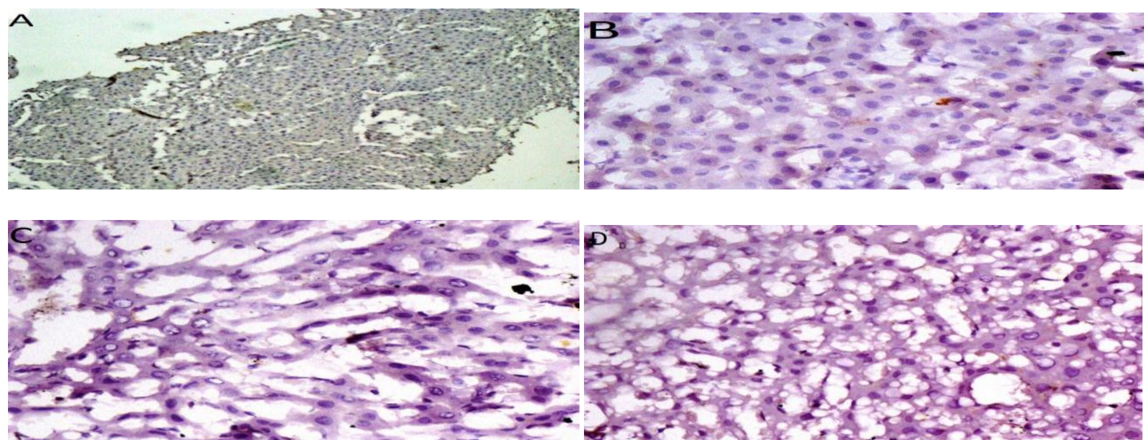

Figure 2: Hepatic Expression of Bcl-2 in rats treated with NDMA and DCLE

This trend was observed in hepatic expressions of Ki-67 and CD15 in the rats. The control group displayed very low expression of Ki-67 (Fig. 3A) while the DCLE group expressed the protein at a mild rate (Fig. 3B). On the other hand, NDMA caused a marked increase in its expression (Fig. 3C), which was drastically reduced by DCLE treatment (Fig. 3D).

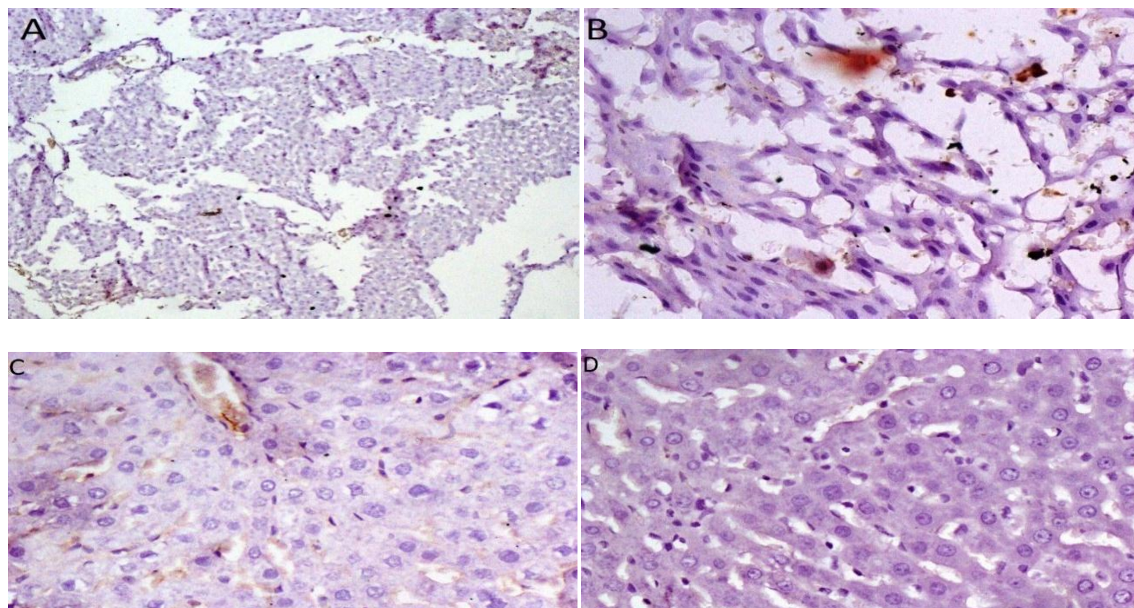

Figure 3: Hepatic Expression of Ki-67 in rats treated with NDMA and DCLE

Similarly, CD15 was mildly expressed in the control and DCLE groups (Fig. 4A and 4B), but NDMA treatment resulted in a marked production of the protein (Fig. 4C)which was reduced in the NDMA + DCLE group to level comparable to the control (Fig. 4D).

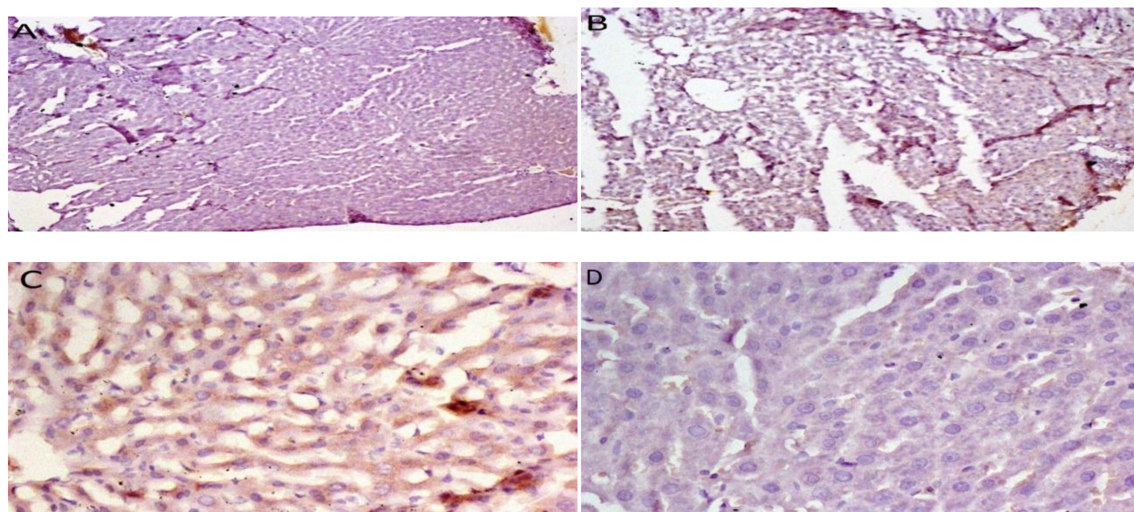

Figure 4: Hepatic Expression of CD15 in rats treated with NDMA and DCLE

Discussion

Human exposures to nitrosable precursors in diets, tobacco smoke and drinking water serve as potential sources of nitrosamines [28, 29, 30, 31]. These compounds have been found to be highly mutagenic and are capable of generating free radicals in living cells [32, 33]. The carcinogenic potential of nitrosamines has been implicated in several cancers [34, 35]. Having discovered a paucity of information on the effects of *Diospyroschloroxylon* on NDMA toxicity, we therefore designed the study to investigate the possible ameliorative potential of this plant in rats intoxicated with NDMA. The data presented show that NDMA elevated weight gain in the rats by 22.8% relative to the control rats.

When the rats were pretreated with DCLE, the weight gain was decreased by 86.7% as against the NDMA-treated rats. However, the treatment has no significant effects on the weight and relative weight of liver of

the rats. In contrast to our observation in the present study, Sivaramakrishnan*et al*. [36] and Singh *et al*. [37] reported body weight loss after administration of *N*-nitrosodiethylamine (NDEA). The NDMA was noticed to significantly increase ($p < 0.05$) the serum and liver protein levels compared with controls, while the pre-treatment with DCLE lowered the levels by 99.1% and 48.7%, respectively, compared with NDMA group. Oxidative stress (OS) occurs when the reactive oxygen species (ROS) are not counterbalanced by the cellular antioxidant defense system, which may lead to the oxidation of DNA, protein or lipid molecules and several events, such as, altered gene expression [38], enhanced cell proliferation [39], genomic or chromosome instability, genetic mutation or tumor development [40, 41]. The activities of both superoxide dismutase and catalase were decreased in the NDMA-treated rats by 99.5% and 47.1%, respectively in the hepatic tissue, whereas a pre-treatment with DCLE was found to elevate the activities by 59% and 89.7%, respectively as against the effect of NDMA. *N*-Nitrosamines such as *N*-Nitrosodibutylamine (NDBA), *N*-Nitrosodiethylamine (NDEA) and NDMA have been observed to reduce the activity of SOD in experimental animals [37, 42]. SOD converts the highly reactive superoxide radical to hydrogen peroxide, which later becomes detoxified by either catalase or glutathione peroxidase [43]. Reduced activities of SOD and catalase could be due to continuous production of superoxide radicals, hydrogen peroxide and other toxic metabolites, which are accumulated, leading to deleterious effects, such as loss of cell membrane integrity, membrane function and oxidative stress [44, 45]. However, a low SOD activity may result in elevated level of superoxide anion which in turn leads to low Nitric Oxide (NO) level, but increased peroxy-nitrite level, due to increased interaction between superoxide anion and nitric oxide. An elevated SOD activity has been noticed in Down's syndrome, while a decreased activity has been observed in diabetes, Alzheimer's disease, rheumatoid arthritis, Parkinson's disease, uremic anaemia, atherosclerosis and thyroid dysfunction [45]. Catalase detoxifies hydrogen peroxide to oxygen and water [46, 47], thereby enhancing acquisition of tolerance to oxidative stress as a form of cellular adaptive response [43]. The level of (GSH) in the hepatic tissue was noticed to significant decrease ($p < 0.05$) in the NDMA group compared with controls. On pre-treatment with DCLE, the level was found to increase by 51.5% when compared with the NDMA-treated rats. Reduced glutathione is a low molecular weight thiol antioxidant, serving as a substrate for glutathione peroxidase (GP_X), an enzyme that detoxifies lipid peroxides and hydrogen peroxide (H_2O_2), associated with oxidative stress. During detoxification of toxicants, GSH is oxidized to glutathione disulfide (GSSG), which is converted back to the reduced form (GSH) by glutathione reductase (GP_R). A reduced GSH level has been associated with low level of cysteine molecules needed for GSH biosynthesis, which in turn may be a result of liver damage [48]. In the course of *N*-nitrosamines metabolism, nitric oxide is produced in excess [9], leading to ROS generation and initiation of neoplasm and tumorigenesis [49, 10, 11]. NDEA- and NDMA-induced carcinogenesis in rats has been associated with GSH depletion [50, 37, 42], which could be as a result of an impairment of GSH synthesis [51]. Pretreatment with GSH before administration of either NDMA or NDEA ameliorated the depletion of GSH and induction of free radicals in mice [50], suggesting that GSH counteracts the toxic effects of these nitrosamines by directly scavenging the free radicals generated. However, GSH could also initiate the repair of oxidative damage [52]. The treatment with NDMA was also observed to significantly elevate ($p < 0.05$) the level of malondialdehyde (MDA) in the rats but pretreatment with DCLE considerably reduced it to a level comparable to the control .*N*-Nitrosamines such as, NDEA [42], NDMA [10] and BBN [9] have been found to elevate MDA level during liver carcinogenesis. Lipid peroxidation (LPO) end-products, including malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) are electrophiles capable of interacting with DNA and proteins to initiate mutagenesis [53]. The MDA forms MDA-DNA adducts which induce frame shift mutations in DNA sequences leading to genetic instability, a process that has emerged as a possible direct link between oxidative stress and human cancinogenesis[54, 55]. The NDMA significantly elevated the DNA fragmentation in the hepatic tissue compared with controls. On pretreatment with DCLE, it was found that DNA fragmentation decreased significantly against NDMA effect in the experimental rats. This result has therefore revealed that NDMA treatment significantly increased DNA fragmentation in the liver relative to control rats, while DCLE pretreatment significantly reduced the level compared with NDMA treatment. The significantly high levels of DNA fragmentation in NDMA–treated rats compared with DCLE-pretreated rats, as observed in the present study, may imply that nitrosamine could cause diseases such as Alzheimer's disease (AD), Parkinson disease, amyotrophic lateral sclerosis and spinal muscular atrophy associated with excessive (pathologic) apoptosis [56]. In addition, dysregulation of apoptosis seems to be directly linked to other human diseases including cancer, autoimmune diseases, and neuronal degeneration [57].

The immunohistochemical analysis revealed that NDMA caused a strong expression of Bcl-2 protein in the liver relative to the control rats, while supplementation with DCLE resulted in moderate expression of the antiapoptotic protein compared with the NDMA-treated rats, The major physiological function of anti-apoptotic Bcl-2 is to maintain an antiapoptotic potential that prevents the action of proapoptotic proteins involved in the formation of mitochondrial membrane permeability transition pore

[58]. In addition to the mitochondrial action, Bcl-2 has also been reported to act at the endoplasmic reticulum (ER), where the antiapoptotic protein modulates Ca^{2+}signalling, thereby promoting proliferation, while increasing resistance to apoptosis [59]. The dynamic balance between Bcl-2 (antiapoptotic) and the proapoptotic members significantly determines whether a cell commits to initiation of apoptosis or not [60, 61].

In the present study, however, the down-regulation of this antiapoptotic protein on DCLE supplementation could be due to the up-regulation of the proapoptotic proteins, antagonizing the Bcl-2 effect. The present study has observed a strong expression of Ki-67 protein, a marker of cell proliferation in clinical studies, in the liver of rats treated with NDMA as against the control rats- However, on supplementation with DCLE, mild to moderate expression of this protein was observed in comparison with the NDMA- treated rats.

The Ki-67 is a nuclear protein being associated with cell proliferation, and usually expressed in every active part of the cell cycle (G_1, S, G_2 and M), but absent in resting (quiescent) cells (G_0) [62, 63]. Although the staining of Ki-67 commonly appears nucleolar or peri-nucleolar, it has also been shown to be associated with ribosomal RNA transcription and inactivation of Ki-67 protein may lead to inhibition of ribosomal RNA synthesis [64]. Ki-67 over- expression is frequently seen in a variety of malignant tissues and is associated with worse survival of individuals with a neuro-endocrine tumour [65].

The result obtained from this study has therefore, indicated the possibility of NDMA to induce malignancy in liver, since a strong expression of Ki-67 was observed in the group treated with this compound, whereas DCLE showed an ameliorative tendency. The immunohistochemical staining of the liver section showed that NDMA caused hyperplasia with high expression of CD15, while a moderate expression was noticed in the rats pre-treated with DCLE.The CD15 antigen (3-fucosyl-N-acetyl-lactosamine) is an extracellular carbohydrate adhesion molecule, belonging to the selectin family, found on the surface of glycoproteins of the plasma membrane of neutrophils and monocytes [66].

This carbohydrate antigen plays a role as a marker of extracellular adhesion and migration of cells. Adhesion molecules are involved in cell-to-cell communication, which is an important factor, not only in tissue development and organization, but also in several other functions in multicellular organisms [67] (Larson and Springer, 1990).

The growth of many cancers depends on self-renewing cells called stem cells or tumor propagating cells (TPCs), to which strong activity of CD15 molecule has been associated [68]. A relationship has been observed between the expression of CD15 antigen and intrahepatic metastasis in hepatocellular carcinoma (HCC) [69]. The present finding may therefore suggest that NDMA could induce metastasis in the hepatic tissue. In conclusion, the present study depicts methanol leaf extract of*Diospyroschloroxylon* displaying potential hepatoprotective property by reversing and stabilizing NDMA-induced disruption of the antioxidant defense system in rats. In addition, the plant extract displayed ability to attenuate DNA fragmentation, reduce cell proliferation and malignancy resulting from NDMA treatment in rats. Further studies are required to elucidate the different phytochemicals responsible for the properties exhibited by *Diospyroschloroxylon*.

References

1. Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for N-Nitrosodimethylamine. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA. 1989.
2. Mitch WA, Gerecke AC, Sedlak DL. *N*-nitrosodimethylamine (NDMA) precursor analysis for chlorination of water and wastewater. Water Res 2003; 37:3733-41.
3. Kumaraguruparan R, Chandra-Mohan KV, Abraham SK, Nagini S. Attenuation of N-meethyl-N*1*-nitro- N-nitrosoguanidine-induced genotoxicity and oxidative stress by tomato and garlic combination. Life Science 2005; 76:2247-55.
4. Jakszyn P, Gonzalez CA. Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence. World J Gastroenterol 2006; 12:4296-303.
5. Boffeta P, Hecht S, Gray N, Gupta P, Straif K. Smokeless tobacco and cancer. Lancet Oncol 2008; 9:667-75.
6. National Toxicology Program. N-Nitrosdimethylamine; CAS no. 62-75-9. Eleventh report on Carcinogens. Public Health Service, U.S. Department of Health and Human Services. 2004. Washington, DC.
7. U.S. Environmental Protection Agency. USEPA, an integrated risk information system for N-nitrosodimethylamine (CASRN 62-75-9). 2006.
8. World Health Organization. N-nitrozamine. Concise International Chemical Assessment Document 38 (CICADS 38) World Health Organization. Geneva, Switzerland. 2002.
9. Oliveira MM, Teixeira JC, Vasconcelos-Nobrega C, Felix LM, Sardao VA, Colaco AA, Oliveira PA, Peixoto FP. Mitochondrial and liver oxidative stress alterations induced by BBN: relevance for hepatotoxicity. J ApplToxicol 2013; 33(6); 434- 43.

10. Farombi EO, Shrotriya S, Surh Y. Kolaviron inhibits dimethylnitrosamine- induced liver injury by suppressing COX-2 and iNOS expression via NF-kB and AP-1. Life Sci. 2009; 84: 149-55.
11. Ghosh D, Choudhury ST, Das N. NanocapsulatedCurcumin: Oral chemopreventive formulation against DEN-induced hepatocellular carcinoma in rat. ChemBiol Inter. 2012; 195(3): 206-14.
12. Hebels DG, Breide JJ, Khampnag R, Kleijans JCS, de Kok TMCM. Radical mechanisms in nitrosamine- and nitrosamide- induced whole genome gene expression modulations in Caco-2 cells. Toxicol Sci. 2010; 116(1): 194-205.
13. Reddy BS, Reddy BP, Raghavulu SV, Ramakrishna S, Venkateswarlu Y, Diwan PV. Evaluation of antioxidant and antimicrobial properties of Soymidafebrifuga leaf extracts. Phytother Res. 2008; 22: 943-947.
14. Higa M, Ogihara K, Yogis N. Bioactive naphthoquinone derivatives from dispyros maritime blume. Chem. Pharm Bull. 1998; 46:1189-93.
15. Kim DSHL, Chen Z, Nguyen VT, Pezzuto JM., Qui SLu ZZ. A concise semi-synthetic approach to betulinic acid from betulin. Synth. Commun. 1997; 27: 1607-12.
16. Thomas RP, Antony AM., Mamen AA. Comparative Phytochemical analysis of DiospyrosChloroxylon leaves in various extracts. Int J Sci Res Pub. 2013; 3(9): 2250-53.
17. Recio MC, Giner RM, Manez S, Gucho J, Julien HR. Investigation of the steroidal antiinflamatory activity of triterpenoids from DiospyrosLeucomelaHostettmannDiospyrosLeucomelas. PlantaMedica. 1995; 61(1): 9-12.
18. Kunle O, Okogun J, Egamana E, Emojevwe E, Shok M.. Antimicrobial activity of various extracts and carvacrol from Lippiamultiflora leaf extract. Journal of Phytomedicine. 2003; 10:59 – 61.
19. Mallavadhani UV, Panda AK, Rao YR. Pharmacology and Chemotaxonomy of Diospyros. Regional Research Laboratory (CSIR), Orissa, India. Phytochemistry. 1998; 49(4):901-51.
20. Santosh MK., Shaila D, Sharanabasappa GK, Seetharam YN, Sanjeevarao I. Phytochemical studies on Bauhinia racemosa Lam. Bauhinia purpuria Linn and Hardwickia binate Rob. E-Journal of Chemistry. 2007; 4(1): 21-31.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall R J. Protein measurement with the folin phenol reagent. J Biol Chem. 1951; 193:265-75.
22. Misra HP, Fridovch J. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1975; 247:3170-5.
23. Aebi H. Catalase in vitro. In: Packer L. Editor. Methods in Enzymology. Orlando FL: Academic Press; pp 121-126. 1984.
24. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by Thiobarbituric acid reaction. Anal Biochem. 1979; 95:351-8.
25. Mitchell JR., Jollow DJ, Potter WZ, Davis DC, Gillette JR, Brodi BB. Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. J PharmacolExer Therap. 1973; 187: 185-94.
26. Wu B, Ootani A, Iwakiri ., Sakata Y, Fujise T, Amemori S, Yokoyama F, Tsunada S, Fujimoto K. T cell deficiency leads to liver carcinogenesis in Azoxymethane-treated rats. ExpBiol Med. 2005; 231:91-8.
27. Chakravarthi S, Long AS, Hannien B, Pasupati T, Palayan K, Talib A. The expression of p53 as a reliable immunohistochemical marker of gastric adenocarcinomas. Res J Med Sci. 2010; 4(1):15-9.
28. Kuhnle GG, Story GW, Reda T, Mani AR, Moore KP, Lunn JC, Bingham SA. Diet-induced endogenous formation of nitroso compounds in the GI tract. Free RadicBiol Med. 2007; 43: 1040-7.
29. Boffeta P, Hecht S, Gray N, Gupta P, Straif K. Smokeless tobacco and cancer. Lancet Oncol. 2008; 9:667-75.
30. Ma F, Wan Y, Yuan G, Meng L, Dong Z, Hu J. Occurrence and source of nitrosamines and secondary amines in groundwater and its adjacent Jialu River Basin, China. Environ Sci Technol. 2012; 46(6): 3236-43.
31. Bara V, Bara C, Bara L. Nitrosamines occurrence in some food products. Ecotoxicologie, ZootehniesiTehnologii de IndustrieAlimentară. 2011; 27-34.
32. Arranz N, Haza AI, Garcia A, Moller L, Rafter J, Morales P. Protective effects of isothiocyanates towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/Hep G2 assay. J ApplToxicol. 2006; 26: 493-99.
33. Arranz N, Haza AI, Garcia A, Rafter J, Morales P. Protective effect of vitamin C towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. ToxicolIn Vitro. 2007; 21:1311–17.
34. Lijinsky W. Chemistry and Biology of N-nitroso compounds; Cambridge Mongraph on Cancer Research: Cambridge University Press: Cambridge. 1992.
35. Knedt P, Jarvinen R, Dich J, Hakulinen T. Risk of colorectal and other gastro-intestinal cancers after exposure to nitrate, nitrite and N-nitrosocompounds: A follow-up study. Int J Cancer. 1999; 80:852-6.
36. Sivaramakrishnan V, Shilpa PNM, Kumar VRP, Devaraj SN. Attenuation of NDEA-induced

hepatocellular carcinogenesis by a novel flavonol, Morin. ChemBiol Inter. 2008; 171(1):79-88.

37. Singh BN, Singh BR, Sharma BK, Singh HB. Potential chemoprevention of NDEA- induced hepatocarcinogenesis by polyphenolics from Acacia nilotica bark. ChemBiol Inter. 2009; 18(1): 20-8.

38. Allen RG, Tresini M. Oxidative stress and gene regulation. Free RadicBiol Med. 2000; 28: 463-99.

39. Toyokuni S. Novel aspects of oxidative stress-associated carcinogenesis. Antioxid Redox Signal. 2006; 8: 1373-7.

40. Ishikawa K, Takenaga K, Akimoto M, Koshikawa N, Yamaguchi A, Imanishi H, Nakada K, Honma Y, Hayashi J. ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. Science. 2008; 320: 661-4.

41. Kumar B, Koul S, Khandrika L, Meacham RB, Koul HK. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phynotype. Cancer Res. 2008; 68: 1777-85.

42. Hussain T, Siddiqui HH., Sheeba F, Vijayakumar M, Rao CV. Evaluation of chemopreventive effect of Fumariaindica against NDEA- and CCl4-induced hepatocellular carcinoma in wistar rats. Asian Pacific J Tropical Med. 2012; 5(8): 623-9.

43. Sankaran M, Vadivel A, Thangam A. Curative effect of garlic on alcoholic liver diseased patients. Jordan J Biol Sci. 2010; 3(4): 147-52.

44. Dahiru D, Obidoa O. Evaluation of the antioxidant effects of ziziphusmauritiana lam: Leaf extracts against chronic ethanol-induced hepatotoxicity in rat liver. Afr J Trad Complement Altern Med. 2008; 5(1): 9-45.

45. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010; 107(9): 1058-70.

46. McCord JM. The evolution of free radicals and oxidative stress. Am J Med. 2000; 108(8): 652-9.

47. Saravanan R, Rajendra N, Prasad NR, Pugalendi KV. Effect of Piper beetle leaf extract on alcohol toxicity in the rat brain. J Med Food.. 2003; 6(3): 261-5.

48. Lauterburg BH, Velez ME. Glutathione deficiency in alcoholics: risk factor for paracetamol hepatotoxicity. Gut. 1988; 29(9): 1153-7.

49. Pradeep K, Mhan CV, Gobianand K, Karthikeyan S. Effect of Cassia fistula Linn. Leaf extract on dimethylnitrosamine-induced hepatic injury in rats. ChemBiol Int. 2007; 167 (1):12-8.

50. Sheweita SA. Glutathione alleviates the influence of N-nitrosamines on the activity of carcinogen-metabolizing enzymes in the liver of male mice. J T U Med Sc. 2006; 191: 72-81.

51. Chandra J, Samali A, Orrenius S. Triggering and modulation of apoptosis by oxidative stress. Free RadicBiol Med. 2000; 29: 323-33.

52. Dorval J, Hontela A. Role of glutathione redox cycle and catalase in defense against oxidative stress-induced endosulfan in adrenocortical cells of rainbow trout (Onorhynchusmykiss). ToxicolApplPharmacol. 2003; 192(2):191-200.

53. Tuma DJ. Role of Malondialdehyde-acetaldehyde adducts in liver injury. Free RadicBiol Med. 2002; 32: 303-8.

54. Zienolddiny S, Ryberg D, Haugen A. Induction of microsatellite mutations by oxidative agents in human lung cancer cell lines. Carcinogenesis. 2000; 21: 1521-6.

55. Valko M, Izkovic M. Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem. 2004; 266: 37-56.

56. Nikitakis NG, Sauk JJ, Papanicolaou SJ. The role of apoptosis in oral disease: Mechanisms, aberration in neoplastic, autoimmune, infectious, haematological and developmental diseases; and therapeutic opportunities. Oral Surg Oral Med Pathol Oral RadiolEndod. 2004; 97: 476-90.

57. Nijhawan D, Honarpour N, Wang X. Apoptosis in neural development and disease. Annu Rev Neurosci. 2000; 23:73-87.

58. Llambi F, Moldoveanu T, Tait SW, Bouchier-Hayes L, Temirov J, McCormick LL, Dillon CP, Green DR. A unified model of mammalian BCL-2 protein family interactions at the mitochondria. Mol Cell. 2011; 44(4): 517-31.

59. Akl H, Vervlossem T, Kiviuoto S, Bittremieux M, Parys JB, De Smedt, Bultynck G. A dual role for the anti-apoptotic Bcl-2 protein in cancer: mitochondrial versus endoplasmic reticulum. BiochimBiophysActa. 2014; 1843 (10): 2240-52.

60. Stresser A, Cory S, Adams JM. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. EMBO J, 2011; 30(18): 3667-83.

61. Garcia-Saez, AJ. The secrets of the Bcl-2 family. Cell Death Differ. 2012; 19(11): 1733-40.

62. Tazzari PL, Bontadini A, Gobbi M, Tassi C, Dinota A, Vasani G, Michieli MG, Pileri S, Baccarani M, Tura S. Comparison of the DNA content, bromodeoxyuridine incorporation and Ki-67 antigen expression in human acute myeloid leukemia. Leuk and Lymphoma. 1990; 3: 45-51.

63. Bruno S, Darzynkiewicz Z. Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Prolif. 1992; 25: 31-40.

64. Rahmanzadeh R, Huttmannn G, Gerdes J, Scholzen T. Chromophore-assisted light inactivation of pKi-67 leads to inhibition of ribosomal RNA synthesis. Cell Prolif. 2007; 40 (3):422-30.

65. Jamali M, Chetty R. Predicting prognosis in gastroentero-pancreatic neuroendocrine tumours: an overview and the value of Ki-67 immunostaining. Endocr.Pathol. 2008; 19:282-8.

66. Stocks SC, Albrechtsen M. Kerr MA. Expression of the CD15 differentiation antigen (3-fucosyl-N-acetyl-lactosamine) on putative neutrophil adhesion molecules CD3 and NCA-160. Biochem J. 1990; 268: 275-80.

67. Larson RS, Springer TA. Structure and function of leukocyte integrins. Immunol Rev. 1990; 114: 181-217.

68. Read TA, Fogarty MP, Markant SL, McLendon RE, Wei Z, Ellison DW, Febbo PG, Wechsier-Reya RJ. Identification of CD15 as a marker for tumor propagating cells in a mouse model of medulloblastoma. Cancer Cell. 2009; 15(2): 135-47.

69. Torri A, Nakayama A, Hrada A, Nakao A, Nonami T, Sakamoto J, Watanabe T, Ito M, Takagi H. Expression of the CD15 antigen in hepatocellular carcinoma. Cancer. 1993; 71: 3864-7.

Source of Support: Nil
Conflict of Interest: None