# Discussion

Diethylnitrosamine initiates hepatocarcinogenesis through the interaction with DNA leading to mutation (**Chakraborty *et al.*, 2007)** and enhanced production of free radicals causing oxidative stress **(Valko *et al.*, 2006).** Promotion using phenobarbital has been implicated in several models of multistage hepatocarcinogenesis as quantified by the increase of number and size of enzyme- altered foci **(Ito *et al.*, 2003)**. On the other hand, a number of investigations is being conducted worldwide, to discover natural products that can hinder the process of carcinogenesis **(Aggarwal *et al.*, 2003; Bishayee, 2012).**

In the current study, the analysis of camel milk showed that the protein % was 3.43 which agrees with previous data showing that protein % of milk of Egyptian camels varied from 3.10 percent **(Farag and Kabary, 1992)** to 3.27 percent (**Bayoumi, 1990).** Moreover the lactose concentration in camel milk was found to be 5 percent which lies within the range from 4.47 % **(Farag and Kabary, 1992)** to 5.53 percent **(Bayoumi, 1990).** Moreover, the percentage of fat in camel milk varies from 0.28 **(Mehaia, 1996)** to 5.70 **(Field et al., 1997)** depending on the hydration status of the animal. In our study the percentage of fat was found to be 5.35 percent indicating good hydration status of camels. Consequently, the high percent of fat, protein and lactose in camel milk used in this study can be attributed to the winter season through which this study was carried out and the good hydration status of camels.

The main components of commercial turmeric are curcuminoids which refer to group of phenolic substances present in turmeric powder **(Wichitnithad *et al.*, 2009).** It was reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds **(Moein and Moein, 2010).** The total phenolic content of ethanolic extract of turmeric in this study was found to be 274.5 mg GAE/ g extract which is comparable to what was reported previously as 260 mg GAE/g **(Sahu and Saxena, 2013).** Therefore the total phenolic content of the turmeric extract can be considered acceptable.

In the present study, there was an increase in body weight of rats injected with DENA (group 2) for the first 14 weeks. It was believed to be due to phenobarbitone administration. Phenobarbitone alone was found to significantly increase the body weight of rats at a dose of 500 ppm and 1000 ppm compared to controls which reflect an increase in the average food intake **(Waterman *et al.*, 2010).** Although the administration of DENA alone for 14 weeks was found to have no effect on the initial and final body weights of rats **(Thapliyal *et al.*, 2003).**

In the current study, the body weight of rats in group 2 (DENA injected rats) showed a significant decrease starting from the $16^{th}$ week. This result agrees with a previous study that also demonstrated a significant decrease in body weight of DENA treated rats compared to control after 16 weeks of treatment **(Arul and Subramanian, 2012).**

In the present study, rats treated with turmeric extract and camel milk showed a significant increase in body weight which could be related to the

turmeric extract. Turmeric acts as a cholagogue, stimulating bile production, thus, increasing the bodies' ability to digest fats, improving digestion and eliminating toxins from the liver **(Akram *et al.*, 2010).** On the other hand, camel milk does seem to contain high levels of insulin or an insulin-like protein which appears to be able to pass through the stomach without being destroyed **(Chaillous *et al.*, 2000).** Therefore, this insulin and insulin like protein and high fat content of camel milk added to the bile stimulating effect of turmeric might have contributed in this weight gain.

In this study, rats in group K treated with cisplatin and L treated with cisplatin and camel milk expressed a significant decrease in body weight at the 32$^{nd}$ week which could be attributed to cisplatin injection. Administration of Cisplatin was found to significantly reduce body weight after 72 hours **(Palipoch *et al.*, 2013).** This decrease can be correlated to various alterations in the small intestine including increased oxidative stress biomarkers, especially lipid peroxidation, changed activities of several enzymatic antioxidants, induced apoptosis of epithelial cells and impaired fluid and electrolyte absorption eventually leading to decreased body weight **(Bearcroft *et al.*, 1999; Chang *et al.*, 2002; Arivarasu *et al.*, 2013).**

Rats in group 2 injected with DENA and administered phenobarbitone revealed a significant increase in the relative liver weight recorded in this study at 19$^{th}$ and 28$^{th}$ week which is in accordance with previous studies **(Thapliyal *et al.*, 2003; Waterman *et al.*, 2010).**

In our study, rats in groups injected with DENA showed no significant difference in relative liver weight at 34$^{th}$ week except for group K treated

with cisplatin. This result could be attributed to cessation of phenobarbitone administration. The significant increase in liver weight in group treated with cisplatin however disagreed with previous studies that showed a significant decrease in relative liver weight in cisplatin treated rats after 15 days **(Arhoghro *et al.*, 2012).** This increase in relative liver weight could be due to the combined effect of DENA as a hepatocarcinogen and the cisplatin as a hepatoxin **(kim *et al.*, 2005).**

At 38$^{th}$ week, rats in group G injected with DENA alone showed a significant increase in relative liver weight whereas rats in group L expressed a significant decrease. The increase in relative liver weight in group G can be attributed to the hepatocarcinogencity of DENA and tumor formation whereas the decrease in group L could be due to the hepatoprotective effect of camel milk **(Darwish *et al.*, 2012).**

The activities of ALT and AST, the indices of liver function showed almost no significant difference between groups at 19 and 28$^{th}$ week of the present study which disapprove with what was recorded previously **(Atakisi *et al.*, 2013)** who reported that diethylnitrosamine induce hepatotoxicity as indicated by elevation in the activity of ALT, AST and ALP detected one week post injection and up to 30 days. The disagreement between our study and previous studies were mainly due to the long time elapse between DENA injection and the serum taken for analysis which is 19 weeks.

Although the serum level of liver enzymes was almost comparable between groups in the present study, the serum albumin expressed a significant decrease in group 2 injected with DENA at 19 weeks post

injection indicating the presence of impaired liver function **(Ibrahim *et al.*, 2008).** Our findings agrees with previous work in which it was reported that a single intraperitoneal injection of DENA depleted the plasma albumin concentration, raised the plasma globulin content, and decreased the ratio of albumin to globulin **(Bishayee *et al.*, 1997; Dai *et al.*, 2013)**. In the present study, the decrease in albumin concentration was transient as there was no significant decrease in serum albumin recorded at 28$^{th}$, 34$^{th}$ and 38$^{th}$ week in the control positive group. It was believed that DENA causes an early and severe inhibition of protein synthesis in hepatocytes which in turn leads to a transient decrease in serum albumin **(Delpino *et al.*, 1984)**

Reduction in serum albumin in group K at 34$^{th}$ week agrees with previous studies that reported a significant decrease in serum albumin following cisplatin treatment **(Saad and Al-Rikabi, 2002).** Hepatic injury induced by chemotherapeutic levels of cisplatin is thought to be a single dose of 5 mg/kg body weight which peaks in about 3 – 5days **(Singh, 1989; Okoko and Oruambo, 2008).**

The AST activity was moderately elevated in group H and highly elevated in group L at 34$^{th}$ week of this study as well as in group J at 38$^{th}$ week. This elevation however was not accompanied with an increase in ALT activity. An isolated elevation of AST values suggests a non-hepatic source of AST which often occurs artefactually due to release of AST from blood cells such as occurs in sample haemolysis. Moreover it could be theoretically due to a reduction in AST clearance or degeneration in other organ like the heart **(Prati *et al.*, 2002).**

In the current study, serum creatinine was elevated in group K and L treated with cisplatin. This result was in accordance with previous studies **(Sener *et al.*, 2012; Ramya *et al.*, 2013).** Experimental studies indicated that the maximum increase in serum creatinine occurs on the fifth day following cisplatin administration **(Francescato *et al.*, 2001).** Our result revealed an increase in blood urea nitrogen in group L as well. It was suggested that the increase in BUN and creatinine during cisplatin toxicity is due to irreversible renal tubular damage **(Dickey *et al.*, 2008).**

In the present study, group E and F treated with cisplatin and was not injected with DENA expressed no increase in creatinine and BUN concentration which imply that DENA is a contributing cause in the elevation of creatinine and BUN in group K and L since it was reported previously that injection of DENA caused a sharp increase in creatinine and BUN **(Khan *et al.*, 2001; Pashmforoosh *et al.*, 2015).**

At $38^{th}$ week of this study there was a significant increase in urea concentration in group L. From the causes of elevation of blood urea is the high protein diet which results in an increase in urea formation by the liver **(Feinfeld *et al.*, 2002).** Moreover the high protein diet especially casein administration in rats was found to induce dietary nephropathy with a subsequent elevation of urea and creatinine which was thought to be due to the impairment of urea and creatinine clearance **(Boorman *et al.*, 1990)**. Consequently this elevation of urea concentration could be attributed to the administration of camel milk in this group.

In the current study, serum level of Alfa fetoprotein of rats in all groups was not highly elevated however it expressed a significant difference. The results were not consistent as there was no difference detected with the control group which disagrees with previous studies **(Liu *et al.*, 2012; Ahmed *et al.*, 2013)** that revealed a significant increase in serum AFP following DENA administration. The reinitiation of AFP synthesis by neoplastic hepatocytes was assumed to be either due to increased transcription of AFP gene or post-translational modification affecting AFP production **(Motalleb *et al.*, 2008).**

Concerning hematological parameters in our study, group G exhibited no significant difference with other groups in estimated parameters at $34^{th}$ week. However, the hemoglobin concentration was reduced later on at $38^{th}$ week. In other studies, DENA treated rats was found to show a decrease in red blood cell count, haemoglobin content, haematocrit value and total white cell count, compared with their normal counterparts **(Bishayee *et al.*, 1997).** This contradiction might be due the alleviation of the direct toxic effect of DENA.

The total leukocytic count showed a significant increase in group K which is believed to be due to cisplatin treatment as it was proved that very low doses of cisplatin causes a significant increase in leukocyte count among which neutrophils and eosinophils recorded a significant increase **(Malarczyk *et al.*, 2003)**. Whereas group L treated with cisplatin and camel milk revealed a normal leukocytic count indicating the ameliorative effect of camel milk on cisplatin induced increase in leukocytic count.

In this study, rats in group G injected with DENA and J injected with DENA and treated with turmeric extract exhibited a significant elevation in MDA concentration (lipid peroxidation level) whereas rats in group K injected with DENA and treated with cisplatin exhibited a significant decrease in SOD activity. Oxidative damage in a cell or tissue occurs when the concentration of reactive oxygen species (O2U, H2O2, and OHU) generated exceeds the antioxidant capability of the cell **(Sies, 1991).** The status of lipid peroxidation as well as altered levels of certain endogenous radical scavengers is taken as direct evidence for oxidative stress **(Khan, 2006).** Oxidative stress plays a central role in diethylnitrosamine induced hepatotoxicity **(Vitaglione *et al.*, 2004).** Diethylnitrosamine was found to cause elevation of lipid peroxidation levels and decreased levels of oxidative stress enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase (GR) and glutathione-S-transferase (GST) in the liver tissue as well as decreased levels of non-enzymic antioxidants like vitamin-C, vitamin-E and reduced glutathione (GSH) shortly after its injection in rats **(Pradeep *et al.*, 2007).** Therefore, the elevation of MDA concentration in group G and group J indicate increased lipid peroxidation and occurence of oxidative stress in these groups which could be attributed to the injection of DENA in both groups and failure of the turmeric extract to posses any antioxidant potential in group J. Moreover, the decrease in SOD activity in group K indicates also the presence of oxidative stress in this group which could be related to the injection of DENA and cisplatin. On the other hand, the SOD activity was high and the MDA concentration was low in group H, I and L which were injected with DENA and treated with camel milk compared to control group. Consequently it could be assumed

that camel milk counteracted the oxidative stress induced by DENA in these groups

The gross findings in present study varied from white or gray foci to large raised parenchymatous mass on the surface of the liver as seen in group J treated with turmeric extract. These gross lesions where believed to be due to the injection of DENA and phenobarbitone administration. DENA is a well known hepatotoxin that induces liver injury and a potent hepatocarcinogen which promotes hepatocellular altered foci and eventually hepatocellular carcinoma **(Boorman *et al.*, 1990; Farber and Rubin, 1991).**

The mean area of altered hepatocellular foci decreased to some degree in all groups at 34$^{th}$ week but was found to be more remarkable in group H, I and L compared to control positive group (G). However, group L recorded the most decrease value. These altered hepatocellular foci which are designated as preneoplastic **(Farber, 1984)** and are anticipated to grow into grossly visible nodules **(Tatematsu *et al.*, 1983).** Since camel milk treatment is the mutual factor between these groups, therefore the decrease in the size of these foci in these groups can be related to the therapeutic effect of camel milk that showed a synergistic effect with turmeric and cisplatin. The ability of camel milk to significantly inhibit the induction of the cytochrome P4501A1 *(Cyp1a1)*, a cancer-activating gene, and to induce the NAD(P)H : quinone oxidoreductase 1 *(NQO1)*, cancer chemopreventive gene **(Korashy *et al.*, 2012a)** in addition to the activation of both the extrinsic and intrinsic apoptotic pathways **(Korashy *et al.*, 2012b)** could be considered the main contributing factors in the improvement seen in these groups added to the antitumor properties of cisplatin **(González *et al.*, 2001)** as seen in group L.

In our study, livers of rats in group G injected with DENA revealed an increase in collagen deposition in the portal area which agrees with previous research work **(El-Shahat *et al.*, 2012; Sun *et al.*, 2012).** Moreover in this study, liver of rats treated with DENA showed hepatocyte vacuolation, hyperplasia of bile duct epithelium and spongiosis hepatis. Liver injury induced by chemicals such as nitrosamines encompasses hepatic vacuolization, necrosis, fibrosis, bile duct or hepatocyte hyperplasia and neoplasia **(Boorman *et al.*, 1990)** which coincided with our findings.

In the present study, livers of rats in group J, group K and some rats in group I at 34$^{th}$ week revealed the presence of peliosis hepatis (sinusoidal dilatation). Peliosis hepatis which is usually multiple and associated with hepatocellular neoplasms was believed to be induced by certain chemicals particularly nitrosamines **(Boorman *et al.*, 1990)**. Although the underlying mechanism of its formation is unclear it was thought to be a result of endothelial damage **(Gushiken, 2000).** Moreover, spongiosis hepatis which is a multilocular cystic lesion containing a finely granular or flocculent eosinophilic material, was reported following exposure to hepatocarcinogen and also could be present within the hepatocellular neoplasms **(Boorman *et al.*, 1990)**. Interestingly, spongiosis hepatis was only seen in groups I, J and K and was severe in group J. It was believed that spongiosis hepatis also known as spongiotic pericytoma is a possible precursor of malignant tumors and was known to be derived from altered perisinusoidal (Ito) cells in rat liver **(Stroebel *et al.*, 1995).** On the other hand, **Karbe and Kerlin, (2002)** indicated that cystic degeneration shouldn't be classified as a preneoplastic or neoplastic lesion.

The peliosis hepatis and spongiosis hepatis were remarkably absent in group H and L as well as in group I at 38th week which would refer to a possible protection inferred with camel milk administration since it's the common factor between these groups.

Hepatocyte vacuolation and sinusoidal dilatation prominently decreased in group H, I and L which could be attributed to the treatment with camel milk as being recorded with **Darwish *et al.* (2012)** who pointed out that decreased degeneration of some hepatocytes following the camel milk feeding period to rats with ethanol induced liver injury were evident.

Moreover these lesions increased in livers of rats in Group K which is believed to be due to treatment with cisplatin therefore emphasizing on the hepatoxic effect of cisplatin on liver exemplified in hepatocyte vacuolation, area of necrosis and infiltration by inflammatory cells **(Arhoghro *et al.*, 2012)**. Camel milk protective effect against the cytotoxicity of cisplatin has been reported previously **(Salwa and Lina, 2010)** which would explain the improvement noticed in group L treated with cisplatin and camel milk compared to group K treated with cisplatin only.

The anti tumor effect of curcumin the active principle of turmeric has been addressed several times in many research work **(Thapliyal *et al.*, 2003; Jagadeesh *et al.*, 2011).** However this anti tumor effect was mainly protection as it was clarified that dietary turmeric mediated protection against DENA induced hepatocarcinogenesis only took place when turmeric was administered during initiation and not after initiation **(Thapliyal *et al.*,**

**2003)**. Therefore the time of administration of curcumin or turmeric is a crucial contributing factor in its effect against hepatocarcinogenesis since it s antitumor effect depends entirely on its antioxidant capacity that counteracts the oxidative stress induced by hepatocarcinogen **(Girish et al., 2009).** Consequently, the negative effect of turmeric extract depicted in group J in our study was mainly due to the delayed administration of turmeric extract in the post initiation phase.

The initiation potential of several carcinogens has been assessed by the induction of preneoplastic placental glutathione s transferase positive foci **(Tsuda *et al.*, 1993).** The relation between the expression of placental glutathione s transferases and hepatocarcinogenesis has been addressed before **(Sato, 1989; Tsuchida and Sato, 1992).** GST-P has been reported as a sensitive marker for preneoplastic hepatic foci during chemical hepatocarcinogenesis in rats **(Qin *et al.*, 1998; 1998; Guo *et al.*, 2000).** GST-P expression in a single hepatocyte promotes cell proliferation as well as its own expression in the adjacent cells **(van Gijssel *et al.*, 1997).** In the current study, the mean percent area of P-GST was best reduced in group L which is treated with camel milk and cisplatin followed by group H and I whereas the least reduction was recorded in group K compared to control group G indicating that camel milk have exhibited a therapeutic effect that was potentiated with cisplatin as evidenced by the reduced mean area of enzyme altered foci.

Hepatoprotection due to feeding on camel milk has been previously documented by **Sharmanov *et al.*, (1982)** who indicated that patients with chronic active hepatitis showed positive shifts in the clinical and laboratory findings after being supplemented with whole camel milk. The anti tumor

effect of camel milk presented in this study can be attributed to its antioxidant properties and scavenging effects on free radicals. Moreover, camel casein was reported to induce apoptosis and in turn reduce the viability of tumor cell lines **(Almahdy *et al.*, 2011).**

In the current study, kidneys of few rats injected with DENA showed tubular hyperplasia and tubular cell adenoma. Although DENA is a potent hepatotoxin and liver is the main target organ, a single dose of DENA was found to have a carcinogenic effect on the renal tubular epithelium causing tubular hyperplasia, tubular cell adenoma and tubular cell carcinoma **(Athar & Iqbal 1998)**. In addition to carcinogenic effect of DENA, a toxic effect summarized in tubular necrosis, inflammatory cells infiltration, thickened tubular basement membranes, glomerular atrophy and tubular casts were also reported in kidneys of rats injected with DENA in the present study and in previous studies **(Sharma and Janmeda, 2013).**

Camel milk administration to rats possessed to some extent an ameliorative effect against Cisplatin induced renal toxicity. In addition the lesions found were comparable to group G injected with DENA only however tubular cell tumors were absent. Kidney lesions recorded in the group treated with camel milk could be related to a renal disease known as protein overload nephropathy or dietary nephritis. Increasing dietary protein especially casein or feeding high caloric diets seems to be the main causes of this condition in rats **(Boorman *et al.*, 1990).** Apparently, this disease is species related as it has not been reported in humans and there were no clear renal-related contraindications to high protein diets in individuals with healthy kidney function **(Friedman, 2004).**

In the present study, kidneys of rats treated with cisplatin whether injected with DENA or not showed acute tubular necrosis, tubular casts, acute interstitial nephritis, thickening of tubular and glomerular basement membrane and chronic interstitial nephritis associated with fibroplastic proliferation, tubular cystic dilatation, eventually fibrosis. These lesions were severe and extensive. Cisplatin is known to cause severe nephrotoxicity. The nephrotoxicity of Cisplatin has been recognized as the most important dose-limiting factor, **(Ramya *et al.*, 2013).** Consequently, these lesions observed in groups treated with cisplatin were attributed to the direct effect of cisplatin.

Different mechanisms has been implicated in cisplatin nephrotoxicity including the expression of glutamyl transpeptidase **(Hanigan *et al.*, 2001)**, the unexpected ability of proximal tubular cells to metabolize cisplatin to a nephrotoxin **(Daley-Yates and McBrien, 1984)**, and the evidence for apoptotic pathways **(Cummings and Schnellmann, 2002)** and reactive oxygen metabolites **(Ueda *et al.*, 2000).**

Although, the data available regarding the effect of turmeric on the kidney lesions observed due to the DENA injection are limited, there was a slight recorded improvement in the groups injected with DENA and treated with turmeric. The lesions found were confined to mild inflammatory cell infiltration with absence of any hyperplastic lesions. The beneficial effect of turmeric against cisplatin induced nephrotoxicity has been documented before **(Ramya *et al.*, 2013).** Therefore, improvement of kidney lesions in the groups injected with DENA and treated with turmeric can be merely attributed to administration of turmeric. The anti oxidant and anti

inflammatory effects possessed by curcumin (the active principle of turmeric) would contributed to the positive effect observed against the oxidative stress exerted by DENA **(Kuo *et al.*, 1996; Jagadeesh *et al.* 2009).**