Effects of Cassia fistula and Ficus carica Leaf Extracts on Hepatocarcinogenesis in Rats

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ABSTRACT

Phytomedicine and uses of some natural bioactive components in prevention and treatment of many diseases, including cancer, have been indicated in many epidemiological and experimental studies. In the present study, the uses of two plant extracts (Cassia fistula and Ficus carica) to inhibit or attenuate the initiation, progression or spread of hepatocarcinogenesis, induced by diethylnitrosamine (DENA) and carbon tetrachloride (CCl4) were examined. DENA was intraperitoneally injected to male albino rats in a single dose (200 mg/kg), two weeks later, animals received a single dose of CCl4 (2 mL/kg) by gavage as 1:1 dilution in corn oil. Subgroups of cancer induced rats were orally treated with methanolic cassia fistula leaf extract and figus carica leaf extract during and after the induction period of hepatocarcinogenesis. The weights of all animals were measured every 2 weeks and the relative weight of liver in all animals was detected after sacrifice by the end of the experimental periods. Blood samples were used to investigate differential white blood cell count and blood serum was used to determine Alkaline phosphatase (ALP), Alpha Fetoprotein (AFP), Carcino embryonic antigen (CEA) and protein kinase C (PKC) as biochemical markers for hepato-cellular carcinoma. The results of this study showed that DENA significantly decreased body weight and increased relative liver weight represented by a significant decrease in ALP, AFP, CEA and PKC. On the other hand, the treatment by leaf extract of cassia fistula and figus carica extract exhibited hepatoprotective effects against DENA and carbon tetrachloride induced cancer in rats. The biochemical findings were confirmed by histopathological and histochemical observations of the liver samples obtained from all groups. It could be concluded that both plant extracts reduced the progress of hepatocarcinogenesis, but figus carica showed more potent effect in prevention and treatment of the disease.

Key words: Diethylnitrosamine, carbon tetrachloride, hepatic carcinogenesis, Cassia fistula, Ficus carica

Introduction

Worldwide, liver cancer is the fifth most common malignancy and the third most common cause of cancer death (Kung et al., 2010). Hepatocellular carcinoma (HCC) is a malignant neoplasm of hepatocytes and constitutes more than 80% of primary malignant liver neoplasms (Satir, 2007). Current knowledge of the mechanisms of cancer suggests that all cancers are both environmental and genetic, meaning that there are multiple causes that involve exposures originating outside the body as well as hereditary or genetic changes that converge to produce the disease (Clapp, 2005).

The major avoidable causes of cancer are: smoking, dietary imbalances, chronic infections and hormonal factors which are influenced primarily by lifestyle, other causal factors in human cancer are excessive sun exposure, viruses (e.g., human papilloma virus and cervical cancer) and pharmaceuticals (e.g. phenacetin, some chemotherapy agents, diethyldithibrostol, and estrogens). (Gold et al., 2002). Chemically-induced cancer generally develops many years after exposure to a carcinogenic agent. Several types of chemicals initiate the carcinogenic process by yielding highly reactive species that bind covalently to cellular DNA. (Valavanidis and Vlachogianni, 2009).

Many hepatocarcinogens such as aflatoxins, acetylaminofluorene3 and diethylnitrosamine have been successfully used to develop hepatocarcinogenesis in animals (Mukherjee et al., 2009). Diethylnitrosamine (DENA) is a potent hepatocarcinogenic nitrosamine, present in cheddar cheese, cured and fried meals, alcoholic beverages, cosmetics, agricultural chemicals and pharmaceutical agents, ground water having high level of nitrate (Mahmoud and Abdul-Hamid, 2012). Nitrate and nitrite are added to meat and fish for the purpose of preservation, as color fixatives and as flavouring (Sadik et al 2009). DENA causes a wide range of tumors in all animal species and considered to be one of compounds which are hazardous to human health (Balamurugan and Karthikeyan, 2012).

Carbon tetrachloride (CCl4) is classified as a possible human carcinogen based on inadequate evidence of carcinogenicity in humans but sufficient evidence in animals. However, there are major deficiencies in the available cancer studies. Animal studies suggest that the carcinogenicity of carbon tetrachloride is secondary to its hepatotoxic effects, indicating a possible threshold (Provincial, 2010). CCl4 is a hepatotoxin, causing liver necrosis, fibrosis and cirrhosis when administered sequentially (Zhang, 2004). Studies in experimental animals...
suggest that the primary cancer risk associated with exposure to carbon tetrachloride is development of liver cancer. CCl4 produced hepatocellular adenomas and carcinomas in rats, mice and hamsters in oral studies and in rats and mice by inhalation exposure (Manibusan, 2010).

Different enzymes and biomarkers are involved in hepatocarcinogenesis depending on the potential cytotoxicity of cancer-causing chemical agents or other factors playing role in the etiology and progression of the disease. From these factors is the metalloenzyme known as alkaline phosphatase (ALP) exists as several tissue specific isoenzymes encoded by separate genes. The enzyme, which is expressed in many species (plants, bacteria and animals) catalyzes the hydrolysis of phosphomonoesters, R-O-PO3, with little regard to the identity of the ‘R’ group (Clifton and Tlentcomb, 2003). Alpha Fetoprotein (AFP) is a tumor associated fetal protein classified as a member of a three domain albuminoid gene family that currently consists of four members: Albumin ALB, vitamin-D3 binding protein (DBP) and alpha-albumin (Mizejewski, 2004).

Carcinoembryonic antigen (CEA) is a member of the immunoglobulin super-family (Thriveni et al., 2007). Protein kinase (PKC) typically phosphorylates serine or threonine residues in basic sequences. In addition to catalyzing phosphorylation reactions, protein kinase C has ATPase and phosphatase activity (Newton, 1995). The p53 gene is one of the tumour suppressor genes that normally prevent uncontrolled multiplication of abnormal cells and experimental findings from the last two decades have established a crucial role for wildtype p53 in intrinsic tumor suppression (Bhatt et al., 2010).

A numbers of modern drugs have been isolated from natural sources and many of these isolations were based on the uses of the agents in traditional medicine. Cassia fistula is an important and potential medicinal plant, a semi-wild Indian labrum that has been used in the treatment of various diseases in different parts of the world (Moshahid et al., 2009). Cassia fistula was reported to show anticancerous activity and actively involved in the pathogenesis of a wide number of diseases including atherosclerosis, cardiac and cerebral ischemia and carcinogenesis. (Bisht et al., 2011). Many Ficus species consist of numerous varieties, significant genetic diversity, outstanding pharmacological activities and these are of remarkable commercial importance. It has been reported that latex and extracts of different speices of ficus are cytotoxic to some human cancerous cell lines. (Chawla, et al., 2012). However, the beneficial effect of medicinal plants are not restricted only to separate pure compounds but also to active extracts fractions or a mixture of both to be very effective in treatment. In this study, the curative and treatment effects of both cassia fistula and ficus carica leaf extracts were studied during and after cancer induction by DENA and CCI4.

Materials and Methods

Animals and experimental design

Fourty adult male albino Wistar rats (110-150g) were housed in polypropylene cages; five animals in each cage at normal room temperature and maintained under 12 h light/dark cycle. The rats were fed pellet diet and water. The animals were acclimatized to laboratory conditions for seven days before commencement of the experiments. Rats were randomly divided into 8 groups.

Group 1: Rats were fed on normal diet and served as normal control for 8 weeks.

Group 2: Cancer was induced by intraperitoneally diethylnitosamine injected in a single dose (200 mg/kg). Two weeks later animals received a single dose of CCI4 (2 mL/kg) by gavage as 1:1 dilution in corn oil.

Group 3: Rats were treated orally with methanolic Cassia fistula leaf extract (500 mg/kg) daily for eight weeks.

Group 4: Rats were treated orally with methanolic Ficus carica leaf extract (500 mg/kg) daily for8 weeks.

Group 5: After injection of DENA and CCI4, rats were treated orally with methanolic Cassia fistula leaf extract as (500 mg/kg) every day for 8 weeks.

Group 6: After injection of DENA and CCI4, rats were treated orally with methanolic Ficus carica leaf extract as (500 mg/kg) every day for 8 weeks.

Group 7: After 8 weeks of DENA and CCI4 were introduced as in group 2, rats were treated orally with methanolic Cassia fistula leaf extract (500 mg/kg) daily for 6 weeks.

Group 8: After 8 weeks of DENA and CCI4 were introduced as in group 2, rats were treated orally with methanolic Ficus carica leaf extract (500 mg/kg) daily for 6 weeks. The weights of all animals were measured every 2 weeks and there lative weight of liver in all animals was recorded after sacrifice.

Relative weight of liver = liver weight /body weight x100.

Materials

DENA, CCI4 and parameters kit were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DENA was dissolved in saline and injected in a single dose (200 mg/kg, i.p.) to initiate hepatic carcinogenesis, while CCI4 was used in a single dose (2 mL/kg) by gavage as 1:1 dilution in corn oil to stimulate liver cell proliferation and regeneration.
Plant extracts

Fresh leaves of *Cassia fistula* were collected during July for preparation of the methanolic leaf extract; washed in tap water, rinsed in distilled water, the leaves were then shade dried for about 1 week and crushed into a coarse powder using a blender. One hundred grams of the coarse powder were kept soaked in methanol (1L, 95%) for 3 days with occasional shaking, then filtered using filter paper. Finally the filtrate was evaporated to dryness under reduced pressure using rotary evaporator and stored until use.

Fresh leaves of *Ficus carica* were collected during August for preparation of the methanolic leaf extract, washed in tap water, rinsed with distilled water, the leaves were then shade dried for about 1 week and crushed into a coarse powder using a blender, two hundred gram of the coarse powder was kept soaked in methanol (1L, 95%) for 3 days with occasional shaking, then filtered using filter paper. Finally, the filtrate was evaporated to dryness under reduced pressure using rotary evaporator and then stored until use.

Analytical procedures

At the end of the experimental periods, animals were sacrificed and blood samples were collected from all animals for differential white blood cell count. Other blood samples were collected in centrifuge tubes and serum was separated from coagulated blood by centrifugation (at 3000 rp.m), stored at -70 until used for biochemical analyses. Liver tissues were collected for histological studies (Bishayee and Chatterjee, 1995) and immunohistochemical studies (Unger et al., 1998).

The biochemical analyses were carried out for determination of alkaline phosphatase (ALP), alpha fetoprotein (AFP), carcino embryonic antigen (CEA) and protein kinase C (PKC) were carried out. Differential white blood cell count was estimated (Dameron et al., 1992). Serum level of ALP was estimated according to (King, 1965) using cusabio kit. AFP was estimated according to (Premalatha and Sachdanandam, 1999) using Enzyme-linked Immunosorbent Assay Kit, Uscn Life Science Inc. CEA was estimated according to (Becker,1990) using ELISA Kit for Rat Carcino embryonic antigen (CEA;CD66) Uscn Life Science Inc and PKC was estimated according to Nishizuka,1992) using rat protein kinase C (PKC) ELISA Kit, cusabio.

Statistical analysis

It was performed using ANOVA test followed by post – hoc which test for multiple comparisons. The results were presented as mean ± standard error. P value 0.05 was considered statistically significant (using SPSS program).

Results and Discussion

Animals body weight

At the beginning of the experiment the mean values of weight in all groups were semi convergent but differences emerged after the passage of time. It was clearly evident from the results that there is a decrease in weight change between DENA group and control group but this decrease was not significant (table 1). After 8 weeks from beginning of the experiment. DENA group showed significant decreased in weight of body comparing to other groups. (fig.1A). The groups treated with the extracts showed changes in weight with the passage of time similar to those of the control group.

Relative liver weight

DENA showed a significant increase in the relative weight of liver compared to control group, mean of control (3.15)g while DENA(4.3)g, while rats of *Cassia fistula* and *Ficus carica* alone recorded 2.6 g and 3.1g. Rats treated with *Cassia fistula* extract and *Ficus carica* extract for eight weeks ranged 2.9g (table 2). Rats treated with *cassia fistula* extract and *Ficus carica* extract after eight weeks showed the same values of *Ficus carica* (fig.1B).

Differential leukocytes count

The carcinogenic group showed a significant decrease in the percentage of lymphocytes compared to the other groups and increased in neutrophils (table 3). DENA show increased in monocytes. It also increased basophils cell which disappear from all groups except the carcinogenic group. *Cassia fistula* and *Ficus carica* increase the mean values of lymphocytes than DENA while extracts decreased the values of monocytes and neutrophils as compared to DENA group (fig.2).

Biochemical results

DENA group showed a significant increase in the mean values of analytical measurements compared to control group as in the ALP , AFP, CEA and PKC (table4). After treatment of carcinogenic rats with *Cassia fistula* extract for 2 months, there are decreasing in the mean values as in ALP , AFP, CEA and PKC. The results showed that there is a decrease in the mean values of groups treated with *Cassia fistula* extract and *Ficus carica* extract after 2 months, both showed a decline more pronounced by lead to significant decreased when
compared with carcinogenic group with evident in groups which treatments immediately after injection with DENA.

Histological and histochemical results

Histopathological examination of liver sections stained with hematoxyline and eosin of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein (fig. 4-6). However, rats treated with DENA alone showed clear signs of severe hepatic injury, the cytoplasm of most hepatocytes appeared vacuolated also destructive damaged cells with hematoma were clear around the central vein. The central vein was surrounded by extensive necrosis and inflammatory infiltrate. It also showed mononuclear cell infiltrates, bile ducts with marked reactive atypia and perportal inflammation with conspicuously dilated blood vessels and ballooning. Degeneration, multinucleated giant cells within some of the granulomas nuclear enlargement, high nuclear, cytoplasmic ratio and prominent nucleoli were also evident (fig. 7-14).

Table 1: Body weight (mean ± S.E.) of control, DENA and CCl4-treated animals and plant extracts treated animals at different periods of treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At the begin of experiment</td>
</tr>
<tr>
<td>Control</td>
<td>140 ±4.47</td>
</tr>
<tr>
<td>DENA+CCl4 group</td>
<td>140 ±4.47</td>
</tr>
<tr>
<td>CAS group</td>
<td>138 ±3.74</td>
</tr>
<tr>
<td>FIC group</td>
<td>142 ±3.74</td>
</tr>
<tr>
<td>DEN+CAS group</td>
<td>134 ±2.4</td>
</tr>
<tr>
<td>DEN+FIC group</td>
<td>144 ±4</td>
</tr>
<tr>
<td>DEN+CAS2 group</td>
<td>132 ±3.7</td>
</tr>
<tr>
<td>DEN+FIC2 group</td>
<td>134 ±5</td>
</tr>
</tbody>
</table>

Table 2: Relative liver weights (mean ± S.E.) of control, DENA and CCl4- treated animals and plant extract- treated animals at the end of experimental period.

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative liver weight relative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>3.150 ±0.104</td>
</tr>
</tbody>
</table>

CAS group: Rats were treated orally with methanolic Cassia fistula leaf extract.
FIC group: Rats were treated orally with methanolic Ficus carica leaf extract.
DENA+CAS group: After injection of DENA and CCl4, rats were treated orally with methanolic Ficus carica leaf extract (500 mg/kg) every day for 8 weeks.
DENA+FIC group: After injection of DENA and CCl4, rats were treated orally with methanolic Cassia fistula leaf extract (500 mg/kg) every day for 8 weeks.
DENA+CAS2 group: After 8 weeks of DENA and CCl4 treatment as in group 2, rats were treated orally with methanolic Cassia fistula leaf extract (500 mg/kg) every day for 6 weeks.
DENA+FIC2 group: After 8 weeks of DENA and CCl4 treatment as in group 2, rats were treated orally with methanolic Ficus carica leaf extract (500 mg/kg) every day for 6 weeks.

* Indicates a statistically significant change compared to control group.
* Indicates a statistically significant change compared to DENA+CCl4 group.

Table 3: Leucocyte differential count % (mean ± S.E.) of control, DENA and CCl4-treated animals and plant extracts treated animals at the end of experiment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lymphocytes%</th>
<th>Neutrophils%</th>
<th>Monocytes%</th>
<th>Eosinophils%</th>
<th>Basophils%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DENA+CCl4 group</td>
<td>CAS group</td>
<td>FIC group</td>
<td>DEN+CAS group</td>
</tr>
<tr>
<td></td>
<td>69 ±2.8</td>
<td>57 ±1.8#</td>
<td>71 ±1.4*</td>
<td>72 ±3.5*</td>
<td>79 ±0.74*</td>
</tr>
<tr>
<td></td>
<td>20.6 ±2.4</td>
<td>21.4 ±0.97</td>
<td>18.6 ±1.0</td>
<td>13.7 ±0.66*</td>
<td>14.8 ±0.58*</td>
</tr>
<tr>
<td></td>
<td>6.8 ±0.37</td>
<td>15.4 ±0.73*</td>
<td>6.8 ±0.74*</td>
<td>7.4 ±0.31*</td>
<td>5.0 ±1.10*</td>
</tr>
<tr>
<td></td>
<td>2.8 ±0.2</td>
<td>3.4 ±0.67#</td>
<td>2.8 ±0.2</td>
<td>1.8 ±0.2</td>
<td>1.2 ±0.2*</td>
</tr>
<tr>
<td></td>
<td>0+0</td>
<td>2+0.2</td>
<td>0+0</td>
<td>0+0</td>
<td>0+0</td>
</tr>
</tbody>
</table>
Liver section of rats treated with *Cassia fistula* extract and *Ficus carica* extract showed more or less normal hepatic cell architectures with normal central vein. This reveals that the extracts are safe on hepatic cells and indicates the non-toxic effect of extracts (fig.15, 16). Liver section of rats treated with *cassia fistula* extract and *Ficus carica* extract for eight weeks after induction of cancer showed disappearing of pyknotic nuclei, vacuoles and degenerated cytoplasm around the central vein only but showed some pyknotic nuclei, intralobular and degeneration cytoplasm intralobular with little dilutions(fig.17,18). Liver section of rats treated with *cassia fistula* and *ficus carica* after eight weeks from induction of DENA and CCl4 showed almost of hepatic cells architectures normal, this mean that the extract of *cassia fistula* and extract of *ficus carica* showed a sign of protection (fig 19,20).

The results of p53 protein expression study on DENA showed positive effect, this indicated that the expression of p53 markedly increased in carcinogenic group (fig.21), but control group effect showed weak positive of p53 protein expression (fig.22). The results of rats treated with *Cassia fistula* extract and *Ficus carica* extract alone showed a weak positive of p53 protein expression + means that extracts are relatively safe for oral administration (fig.23,24). Liver sections of rats treated with *Cassia fistula* extract and *Ficus carica* extract for eight weeks showed a weak positive effect of p53 protein expression (fig.25,26). Liver sections of rats treated with *Cassia fistula* extract and *Ficus carica* extract after eight weeks from induction of DENA and CCl4 revealed reduction of the positive effect of P53 expression (fig.27,28).

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**Table 4:** Biochemical parameters of serum (mean ±S.E.) of control, DENA and CCl4-treated animals and plant extracts-treated animals at the end of experiment.  

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group</th>
<th>DENA+CCl4 group</th>
<th>CAS group</th>
<th>FIC group</th>
<th>DEN+CAS group</th>
<th>DEN+VIC group</th>
<th>DEN+CAS2 group</th>
<th>DEN+VIC2 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>124.8±11.499</td>
<td>288±11.954#</td>
<td>187±2.510*</td>
<td>188±4.415*</td>
<td>228±14.934#</td>
<td>225±14.972*</td>
<td>186±11.043*</td>
<td>173±22.07*</td>
</tr>
<tr>
<td>Alpha fetoprotein (ng/mL)</td>
<td>0.4980±0.1080</td>
<td>2.36±0.2163#</td>
<td>1.28±0.1781*</td>
<td>1.23±0.1761*</td>
<td>2.04±0.2634</td>
<td>2.08±0.1342</td>
<td>1.49±0.2554*</td>
<td>1.54±0.2993*</td>
</tr>
<tr>
<td>Carcinogenic embryonic antigen (ng/mL)</td>
<td>0.5180±0.1003</td>
<td>3.46±0.3789#</td>
<td>1.6520±0.1575*</td>
<td>1.5940±0.1400*</td>
<td>2.6360±0.2883</td>
<td>2.3420±0.2176*</td>
<td>1.5980±0.1398*</td>
<td>1.6300±0.2315*</td>
</tr>
<tr>
<td>Protein kinase (pmol/L)</td>
<td>0.5488±0.139</td>
<td>6.6120±0.356#</td>
<td>3.7316±0.416*</td>
<td>2.6340±0.193*</td>
<td>4.8502±0.664</td>
<td>4.3902±0.549*</td>
<td>3.8972±0.878*</td>
<td>4.9842±1.1259</td>
</tr>
</tbody>
</table>

#: Indicates a statistically significant change compared to control group.  
*: Indicates a statistically significant change compared to DENA+CCl4 group.

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Fig. 4: Photomicrograph of liver section of control rat stained with H&E (X400) showing normal lobular appearance having normal central vein (C.V), centrally located nucleus of cells (long arrow) and normal radiating cords of hepatocyte (short arrow).
Fig. 5: Photomicrograph of liver section of control rat stained with H&E (X400) showing normal central vein (long arrow) and centrally located nucleus of cells (long arrow) and normal radiating cords of hepatocyte (short arrow).

Fig. 6: Photomicrograph of liver section of control rat stained with H&E (X400) showing normal nucleus of hepatocyte.

Fig. 7: Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 stained with H&E (X200) showing central vein (c.v) surrounded by extensive necrosis and inflammatory infiltrate.
**Fig. 8:** Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 stained with H&E (X400) showing areas of aberrant hepatocellular phenotype with variation in nuclear size (long arrow) and irregular hepatocytes (short arrow).

**Fig. 9:** Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 stained with H&E (X400) showing the mononuclear cells infiltrate bile duct (long arrow) with marked reactive atypia (short arrow).

**Fig. 10:** Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 stained with H&E (X400) showing periportal inflammation with conspicuously dilated blood vessel (long arrow) and ballooning degeneration (short arrow).
Fig. 11: Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 stained with H&E (X400) showing nuclear enlargement, high cytoplasmic ratio (short arrow) and prominent nucleoli (long arrow).

Fig. 12: Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 stained with H&E (X400) showing multinucleated giant cell (long arrow) within some of the granuloma (short arrow).

Fig. 13: Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 treated with DENA stained with H&E (X400) showing abnormal nuclei of hepatocytes.
Fig. 14: Photomicrograph of liver section of carcinogenic rat induced by DENA and CCl4 stained with H&E (X 100) showing destructive cells with hematoma around central vein.

Fig. 15: Photomicrograph of liver section of rat treated with Cassia fistula extract stained with H&E (X 400) showing normal hepatocytes, normal central vein (C.V) with normal centrally located nucleus of cells.

Fig. 16: Photomicrograph of liver section of rat treated with Ficus carica extract stained with H&E (X 400) showing normal central vein, normal radiating cords of hepatocyte (short arrow) and centrally located nucleus of cells (long arrow).
Fig. 17: Photomicrograph of liver section from rat treated with *Cassia fistula* extract for 8 weeks stained with H&E (X 400) showing normal central vein (C.V), normal radiating cords of hepatocytes (short arrow) and centrally located nucleus of cell (long arrow).

Fig. 18: Photomicrograph of liver section from rat treated with *Ficus carica* extract for 8 weeks stained with H&E (X 400) showing normal central vein (C.V), centrally located nucleus (long arrow) and normal radiating cords hepatocyte (short arrow).

Fig. 19: Photomicrograph of liver section from rat treated with *Cassia fistula* extract after 8 weeks stained with H&E (X 400) showing normal central vein (C.V) and most of cells having normal nuclei (long arrow) and normal radiating cords of hepatocytes (short arrow).
Fig. 20. Photomicrograph of liver section from rat treated with *Ficus carica* extract after 8 weeks stained with H&E (X 400) showing normal central vein (C.V) and most of cells having normal nuclei (long arrow) and normal radiating cords of hepatocytes (short arrow).

Fig. 21: Photomicrograph of liver section from control rat immunostained with p53 (X400) showing weak positive immunostaining of hepatocytes nuclei.

Fig. 22. Photomicrograph of liver section from carcinogenic rat induced by DENA and CCl4 immunostained with p53 (X400) showing strongly positive expression of hepatocytes nuclei.
Fig. 23: Photomicrograph of liver section from rat treated with *Cassia fistula* extract immunostained with p53 (X 400) showing weak positive immunostaining of hepatocytes nuclei.

Fig. 24: Photomicrograph of liver section from rat treated with *Ficus carica* extract immunostained with p53 (X400) showing weak positive immunostaining of hepatocytes nuclei.

Fig. 25: Photomicrograph of liver section from rat treated with *Cassia fistula* for 8 weeks immunostained with p53 (X400) showing weak positive expression of P53 in hepatocytes nuclei.
Fig. 26: Photomicrograph of liver section from rat treated with *Ficus carica* for 8 weeks immunostained with p53(X400) showing weak positive expression of P53 in hepatocytes nuclei.

Fig. 27: Photomicrograph of liver section from rat treated with *cassia fistula* extract after 8 weeks immunostained with p53 (X400) showing reduction of the positively of P53.

Fig. 28: Photomicrograph of liver section from rat treated with *ficus carica* after 8 weeks immunostained with p53(X 400) showing reduction of the positively of P53.
Discussion

Liver cancer causes a decrease in body weight (Mahmoud and Abdul-Hamid, Bishayeea et al., and Wu, X. et al. 2012, 2010, 2001). Proliferation of cells in the liver tissue induced by DENA and CCl4 was evident from the increase in liver weights (Kartika, 2010). In the present study, DENA decreased the ratio of body weight. On the first week following DENA injection, the rats began to show a slow growth compared to the control and plant-treated groups. The final weight showed a significant decrease when compared with control group. Anorexia appeared in advanced cancer (Davis, et al 2004). Involuntarily weight loss is characteristic of solid tumors (Inui, 2002). Glucose used by tumors is inefficient because most cancers are hypoxic and are unable to metabolize glucose fully to CO2. Tumors use glucose as a primary source of energy and generate a large amount of lactate which is converted to glucose in the liver by way of the Cori cycle (Tisdale, 2000). The generation of glucose from lactate in the process of gluconeogenesis requires six ATP molecules which is energy inefficient; this accounts for some of the high resting energy expenditures in cancer. Resting energy expenditures are increased in cancer, particularly when energy expenditures are corrected for lean body mass (Ruth et al., 2005). Increased sympathetic activity in cancer causes increased resting energy expenditures (Argiles et al., 2003).

Liver cancer causes increase in the relative weight of liver (Bishayeea et al., Kartika et al., 2010 and Wu et al., 2001). In this study, the carcinogenic group has increased liver weight. This means that diethyl nitrosamine increased the weight of liver, this may be due to increase in the percentage of water in the liver. Glycogen and fat, as pointed represent passive reserve material, while an increase in protein content, they believe, reflects work performed by the organ. It is possible that similar work on the part of the liver concomitant with the synthesis of neoplastic tissue may account for the increase in the weight of the organ. (Eleanor, 1948).

The immune system conducts surveillance and eradicates infections and controls early malignant growth. However, in clinical cases the cancer evades the immune system resulting in progression of the malignancy most likely leading to death of the patient. Defective functions of the immune system are thought to be an important mechanism by which tumors escape from the immune surveillance (Kvistborg, 2009). Present study showed that DENA decreased the number of lymphocytes. Lymphocytes constitute one of the most important effective mechanisms of anti-tumor-immunity. In order for CD8+ T cells to recognize antigens, these need to be exposed on the tumor cells in association with the human leukocyte antigen (HLA) class I proteins (Deschoolmeester, 2012).

It was found that neutrophils promote cancer cell adhesion within liver sinusoids and thereby influence metastasis. Neutrophil depletion prior to cancer cell inoculation resulted in a decreased number of gross metastases in an intra-splenic model of liver metastasis. This effect was reversed when inflamed neutrophils were co-inoculated with cancer cells. In addition, early adhesion within liver sinusoids was inhibited in the absence of neutrophils and partially restored with a short perfusion of isolated activated neutrophils. Intra-vital microscopy showed that cancer cells adhered directly on top of arrested neutrophils, suggesting that neutrophils may act as a bridge to facilitate interactions between cancer cells and the liver parenchyma (Jonathan, 2012).

In the present investigation, the use of DENA increases number of monocytes in carcinogenic group. In HCC patients, soluble factors derived from hepatoma cells, including extracellular matrix components, effectively induced the formation of tumor-associated Mφs (TAMs). Interestingly, kinetic analysis revealed 2 opposing functional stages in the TAM life cycle; monocytes are rapidly activated during a narrow time window, 4 to 16 hours after their first exposure to hepatoma cell culture supernatants, and afterward the same cells become exhausted and their production of cytokines is extinguished, with the exception of IL-10 (Kuang, 2009).

During carcinogenesis, some enzymes can be used as biochemical indicators of tumors. Elevation of alkaline phosphatase is one of the signs, suggesting space occupying lesions in the liver. Alp in blood serum increased in liver cancer (Al-Rejaie et al., 2009; Ibrahim and Sahin, 2009, 2008, 2005). In this study, ALP was increases in carcinogenic group. An increased activity of ALP was seen in blood serum and liver of animals with HCC, this may be due to the disturbance in secretory activity or due to altered gene expression in these conditions (Sayed-Ahmed et al., 2010). It is known that liver cancer increases in serum AFP (Kew, Mohamed, and Parikh, 2012, 2012, 2007) and CEA (Moshahid et al., Sturgeon et al and Zhou et al., 2009, 2008, 2007). In present study, DENA stimulated the increase in level of AFP and CEA. Elevation of serum AFP levels has been reported in several diseases including HCC. AFP along with CEA is most extensively used in the diagnosis of HCC. In previous studies there was an increased level of AFP and CEA in the carcinogen administered animals confirming the presence of HCC (Sahin, 2005). The serum AFP measurements may be useful as a sensitive marker system for the early detection of recurring hepatocellular carcinoma, even before the clinical symptoms are evident. (Malati, 2007). High levels of CEA are associated with advanced stage of the disease. CEA elevations accompany both benign and malignant diseases of liver. Increased CEA level signifies the progression from benign to malignant.
transformation (Mittal et al., 2012). It has been also reported that CEA is secreted into the serum in high level of adenocarcinoma of digestive epithelia. CEA increases with an increase in the size of tumor (Jahan, 2011).

Protein kinase C (PKC) was reported to have a pivotal role in carcinogenesis. The level of serum PKC was increased in liver cancer (Aaltonen et al., 2006, 1997). In the present study, DENA increased PKC in blood serum. A correlation between PKC activation and/or expression and the ability of tumor cells to form metastasis has been observed. PKC activity was demonstrated to be altered in certain malignancies. One important concomitant of PKC activation is the intracellular redistribution of the enzyme from the cytosol to the plasma membrane and also to the nucleus. Both α and β isoforms of PKC are the most of pkc isoforms deeply implicated in carcinogenesis and metastasis (Tessitore and Comolli, 1997). PKC isoenzymes have been shown to display variable expression profiles during cancer progression, depending on the particular cancer type. Activation of different PKC isoenzymes has been shown to result in distinct cellular responses. Sustained activation of PKC has been suggested to induce proliferation, differentiation, apoptosis, migration, or tumorigenesis (Aaltonen, 2006).

Diethyl nitrosamine, one of the most important environmental carcinogens, has been suggested to cause the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury. As liver is the main site of diethyl nitrosamine metabolism, the production of ROS in liver may be responsible for its carcinogenic effects. The involvement of oxidative stress in diethyl nitrosamine induced hepatotoxicity and carcinogenicity emphasizes the need for development of novel compounds with potent antioxidant activity (Mahmoud and Abdul-Hamid, 2012). Carbon tetrachloride (CCl4) is a hepatoxina, causing liver necrosis, fibrosis and cirrhosis when administered sequentially (Zhang, 2004). The histological changes of liver induced by DENA and CCl4 in this study may be attributed to their oxidative properties; formation of radicals and mutagenic acting which initiate carcinogenesis.

Hepatoprotective activity of any drug is the ability of its constituents to inhibit the aromatase activity of cytochrome P 450, thereby favoring liver regeneration. On that basis, it is suggested that flavonoids in cassia fistula could be a factor for exhibiting the hepatoprotective activity (Wasu and Muley, 2009). In the present study the treatment by leaf extract of Cassia fistula and Ficus carica exhibited hepatoprotective effects against DENA and carbon tetra chloride induced cancer in rats. A possible mechanism of the Ficus carica extract as hepatoprotective may be due to its anti-oxidant effect or inhibition of cytochrome P450 which impair the bioactivation of CCl4 into their corresponding reactive species (Krisha et al., 2007). The antitumor effects of plant flavonoids have been reported to induce cell growth inhibition and apoptosis in a variety of cancer cells.

It was found that Cassia fistula leaf extract and Ficus carica leaf extract contain: rhein, physcion, epicatechin, luteolin, chrysin, apigenin, quercetin and polyphenols. Quercetin is a ubiquitous bioactive flavonoid, which can inhibit the proliferation of cancer cells. It was reported that quercetin may be a potential chemopreventive or therapeutic agent in hepatocarcinoma cells and further efforts to investigate these possibilities are needed (Seufi et al., 2009). Rutin is a polyphenolic natural flavonoid which possesses antioxidant and anticancer activity. The hepatoprotective effect of rutin was evaluated against carbon tetrachloride CCl4-induced liver injuries in rats. Rutin showed significant protection with the depletion of ALP (Khan, 2012).

Luteolin displays specific anti-inflammatory and anti-carcinogenic effects, which can only partly be explained by its anti-oxidant and free radical scavenging capacities. When compared to other flavonoids, luteolin was usually among the most effective ones, inhibiting tumor cell proliferation. (Seelinger, 2008). It can delay or block the development of cancer cells in vitro and in vivo by protection from carcinogenic stimuli, by inhibition of tumor cell proliferation, by induction of cell cycle arrest and by induction of apoptosis via intrinsic and extrinsic signaling pathways (Batra, 2013). Rhein is a primary anthraquinone found in herb and has been shown to have some anticancer effects. (Li, 2012). Chrysin is a flavone, although, it was found to significantly sensitize the TNF alpha-induced apoptosis in human colorectal cancer cell line HCT-116, human liver cancer cell line HepG2 and the human nasopharyngeal carcinoma cell line CNE-1 (Khoo, 2010). A possible mechanism for the potential anti-carcinogenic effects of flavonoids could be their ability to inhibit various PKs, thereby inhibiting signal transduction event of cell proliferation. Recently, PKC was shown to be efficiently inhibited by flavones and flavonols (Batra, 2013).

Hepatocellular carcinoma is defined by the World Health Organization as a malignant tumor composed of cells resembling hepatocytes but abnormal in appearance; a plate-like organization around sinusoids is common and is nearly always present somewhere in a tumor (Alison and Lovell, 2005). The histological changes described in the present investigation as a result of DENA administration. (Mahmoud, Al-Rejaie and Ibrahim, 2012, 2009, 2008) induced severe hepatic necrosis and DNA damage in liver following administration of the identical dose of DENA to rats. In addition to liver damage, DENA can alkylate DNA molecule with itself being converted to highly reactive molecule by P-450 dependent oxygenases. (Mahmoud and Abdul-Hamid, 2012).

The expression of p53 was significantly increased in HCC patients before treatment (Kartika, Moshahid and Jaworski, 2010,2009, 2005). In this study, DENA group showed strongly positive expression of p53 in hepatocytes nuclei. This may be due to that: These gene products normally modulate biochemical pathways that
regulate cell death and cell proliferation. Deregulation of signaling pathways during the development of hepatocellular carcinoma affects normal cellular processes such as cell cycle and apoptosis (Mukherjee, 2009). Somatic mutations of the tumor suppressor p53 gene are the commonest genetic abnormality in human cancer and evidence supports a high level of p53 alterations in HCC (Gomaa, 2008).

It is well known that inactivation of p53 is the most common genetic alteration in human cancer including HCC. The p53 has a critical role in regulation of cell cycle, DNA repair and synthesis as well as in apoptosis. The prevention of cancer is profoundly dependent on the p53 tumor suppressor protein. The ability of p53 to eliminate excess, damaged or infected cells by apoptosis is vital for the proper regulation of cell proliferation. Cassia fistula and Ficus carica extracts make reduction of the positive expression of P53, this mean these extracts may be protect liver against carcinogenic effect of DENA and CCl4 (Batra, 2013).

Liver sections of rats treated with Cassia fistula and Ficus carica showed improved hepatocellular architecture with signs of recovery, indicating the protective effect of plants, similar to that of control treatment. Further, individual treatments of extract alone did not cause a changes in the biochemical parameters, as well as pathological observations indicating their non-toxic nature.

The results of this study showed that the leaf extracts of Cassia fistula and Ficus carica are almost in accordance with the previous results and exhibit a good hepatoprotective effect against DENA induced hepatotoxicity in rats. However, Ficus carica showed more potent effect in prevention and treatment of the disease. A combination of both plant extracts may provide more desired activities to cure hepatocarcinogenesis. This may require more investigations to find out the most efficient and satisfactory results of this herbal combination.

References


