Materials and Methods

3.1. Animals

Ninety six male Wister rats, weighing 100- 120 g, were purchased from the animal house of the National Research Center (El Dokki, El Giza, Egypt). This experimental work was approved by Ethics of animal use in research committee (EAURC), Faculty of Veterinary Medicine, Cairo University, Egypt. The animals were housed in metal wire mesh cages (4- 5 rats per cage) and were left for two weeks before beginning the experiment for acclimatization. The housing conditions including temperature 25± 2 °C, relative humidity 50-60%, and 12 hour photoperiods were set. The rats were supplied with a pelleted diet and water *ad libitum*.

3.2. Chemicals

3.2.1. Diethyl nitrosamine (DENA) was 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.) for initiation of hepatocarcinogenesis.

3.2.2. Phenobarbitone was kindly supplied by the Egyptian international pharmatheutical industry Co. (EIPICO). Phenobarbitone was dissolved in the drinking water at a concentration of 500 ppm for tumor promotion.

3.2.3. Folin-Ciocalteau Phenol TS was purchased from Science Lab.com (U.S).

3.2.4. Cisplatin was purchased from El Azaby under the commercial name of Cisplatin 10 mg.

3.2.6. Tween 20 was purchased from Nice chemicals Pvt, Ltd. (India). All other chemicals used were of high grade quality.

3.3. Camel milk

Camel milk was obtained from two different sources for comparison. Marsa Matrouh camel milk was purchased from the market. Camel milk was also purchased from a camel ranch in Ras Sedr, south of Sinai. The Ras Sedr camel milk was chosen to be used in our experimental study. Camel milk was analyzed using Milk Lactoscan in department of nutrition, Faculty of Veterinary Medicine, Cairo University. All analytical studies were performed in triplicates.

3.4. Ethanolic extract of Curcuma longa

Curcuma longa were purchased from the market (Haraz market) as rhizomes then subjected to grinding into fine particles. The plant was weighed and homogenized in 95 % ethanol at a ratio of 1:5 of plant to ethanol and left to soak for 3 days at 25 °C with occasional shaking and stirring. The mixture was then filtered and the resulting liquid was concentrated under reduced pressure at 45 °C in a rotary evaporator to yield a dark gummy yellow extract. The concentrated extract was then kept in the incubator at 45 °C for 7 days to evaporate the ethanol residue yielding the crude rhizome extract. Extracts were then dissolved in 10 % tween 20 before being orally administered to animals in concentration of 250 mg/kg body weight **(Salama *et al.*, 2013).**

3.5. Total phenol content of ethanolic extract of Curcuma longa

Total phenolic content was determined with the Folin-Ciocalteu reagent according to a procedure described by **Singleton and Rossi (1965).** The ethanolic extract of curcuma longa (1 mg) was first dissolved in 1ml dimethyl sulfoxide (DMSO). Next, 20 µl of the extract was added into 100µl

of Folin-Ciocalteau reagent, and the resulting mixture was incubated in the dark for 3 min. then, 100 µl of sodium carbonate (1 g/10ml) solution was added to the mixture and mixed thoroughly. The final mixture was kept in the dark for 1 hr and its absorbance (750 nm wavelength) was read by a UV spectrophotometer. All procedures were carried out in triplicate. Linear standard curve were produced by serial dilution of gallic acid (1 mg/ ml DMSO) and the absorbance was read at 750 nm. The results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of the ethanolic extract of curcuma longa.

3.6. Experimental design

Ninety six male rats were divided into two groups (48 each). Group 1 was an untreated control. Group 2 was injected intraperitoneally with a single dose (200mg/kg body weight) of diethylnitrosamine dissolved in saline to initiate hepatocarcinogenesis. After one week, phenobarbitone was added to drinking water of group 2 at a concentration of 0.05% (500 ppm) for 28 weeks. After 28 weeks, each group was subdivided into six groups. Group A served as a non treated control. Group B received camel milk (5 ml) daily by oral intubation. Group C received both camel milk (5 ml) and curcuma long ethanolic extract (250 mg/kg) daily by oral intubation. Group D received ethanolic curcuma longa extract (250 mg/kg) daily by oral intubation. Group E was intraperitoneally injected with cisplatin (5 mg/kg) for 2 times with 3 weeks interval. Group F was injected with Cisplatin and recieved Camel milk via oral intubation. Group 2 G, H, I, J, K and L received similar treatments as group 1 **(Table 1).**

Table (1): showing the experimental design

	Phase I Induction of liver cancer	Phase II treatment	
Group 1	- ve	Group A	Non treated negative control
		Group B	Treated with camel milk
		Group C	Treated with camel milk and curcuma longa extract
		Group D	Treated with curcuma longa extract
		Group E	Treated with Cisplatin
		Group F	Treated with cisplatin and Camel milk
Group 2	+ve*	Group G	Non treated positive control
		Group H	Treated with Camel milk
		Group I	Treated with camel milk and curcuma longa extract
		Group J	Treated with curcuma longa extract
		Group K	Treated with Cisplatin
		Group L	Treated with cisplatin and Camel milk

* injected I/P with 200 mg/kg body weight DENA and received phenobarbitone in drinking water after one week at a concentration of 500 ppm for 28 weeks.

3.7. Body weight

The body weight of rats was recorded weekly throughout the experimental period.

3.8. Sampling

Euthanasia was performed four times throughout the experimental period in which two times were carried out before initiation of treatments to asses the development of altered hepatocellular foci and cancer and two times after initiation of treatments. The first and second euthanasia were carried out at 19^{th} and 28^{th} week of DENA injection and phenobarbitone administration respectively. The third and fourth euthansia were carried out at 34^{th} and 38^{th} week from DENA injection. Three rats from each group and subgroup were euthanized at each time. The euthanasia was performed by exposing the animals to an overdose of diethyl ether.

3.8.1. Blood sample

Before euthansia, blood samples were collected from the medial canthes of the eye using capillary tube from all rats. The serum was separated from coagulated blood by centrifugation at 3000 rpm for 10 min and two aliquots were made for each sample. The serum was stored at -20 C° until being assayed. Whole blood was collected in ready to use tubes containing EDTA.

3.8.2. Tissue sample

Tissue samples from liver and kidney were fixed in 10% neutral buffered formalin for histopathology. Also frozen liver spicemen were collected for assessing the oxidative stress enzymes.

3.9. Relative liver and kidney weight

At euthansia, both the liver and kidney weights were also recorded in order to calculate the relative liver and kidney weight using the following equation:

Relative organ weight = organ weight / body weight * 100

3.10. Hematological examination of the collected blood samples

The packed cell volume, count of RBCS, concentration of hemoglobin, total leukocytic count and differential leukocytic count were determined in non coagulated blood. Packed cell count was determined by microhaematocrit technique. Red blood corpuscles count and total leukocytic count were performed using haemocytometer. Haemoglobin concentration was estimated by cyanohaemoglobin method using Drabkin's solution **(Jain, 2000).**

3.11. Biochemical assay

3.11.1. Liver function tests

3.11.1.1. Alanine aminotransferase (ALT/GPT)

The ALT enzymatic activity was assayed spectrophotometrically in the serum of all rats according to **Reitman and Frankel (1957)** using spectrum diagnostic kit (Egypt).

3.11.1.2. Aspartate aminotransferase (AST/ GOT)

The AST enzymatic activity was assayed spectrophotometrically in the serum of all rats according to **Reitman and Frankel (1957)** using Spectrum diagnostic kit (Egypt).

3.11.1.3. Serum Albumin and total protein

The concentration of albumin and total protein in serum of all rats was assayed using Diamond kit (Egypt). Serum total proteins were determined by Biuret reaction using copper ion in alkaline media according to **Weichselbam (1946).** Serum albumin was determined according to **Bartholomew and Delaney (1966)** in which bromocresol green binds with albumin at PH 4.2.

3.11.2. Serum α- fetoprotein

The concentration of α- fetoprotein in serum was assayed according to **Gibbs *et al*. (1987)** using rat alpha-fetoprotein ELISA kit (WKEA Med Supplies Corp., China).

3.11.3. Kidney function tests

3.11.3.1. Urea

The concentration of urea in the serum of all rats was assigned using Spectrum kit (Egypt) according to **Lile *et al*. (1957**).

3.11.3.2. Creatinine

The concentration of creatinine in the serum of all rats was assigned using Spectrum kit (Egypt) according to **Fabiny and Eringhausen (1971)** in which the creatinine is coupled with picric acid.

3.11.4. Estimation of superoxide dismutase

The activity of superoxide dismutase was estimated in the liver homogenate made from frozen liver samples using Biodiagnostic Kit (Egypt). The superoxide dismutase activity was measured as the degree of inhibition of auto-oxidation of pyrogallol in an alkaline pH according to the method described by **Marklund and Marklund (1974).**

3.11.5. Estimation of MDA (Lipid peroxidation)

The concentration of Malonaldhyde (MDA) in the liver homogenate was estimated using Biodiagnostic kit (Egypt). The lipid peroxides content in the liver homogenate was estimated by monitoring the thiobarbituric acid reactive substance formation as described by **Ruiz-Larrea *et al*. (1994)**

3.12. Gross and Histopathological studies

The rats were observed throughout the experimental period for any clinical signs. At slaughter time, the rats were subjected to thorough postmortem examination. Liver and kidney samples were fixed for 48 h in 10% neutral buffered formalin, dehydrated by passing successfully in ascending concentrations of ethyl alcohol, cleared in xylene and embedded in paraffin. Sections (5–6 μm thick) were prepared and stained with H&E stain for microscopic examination. Special stains such as PAS and MTC were used **(Bancroft *et al*., 2012).** Liver specimen from three different lobes were examined for each rat and lesion score was performed. Liver cell foci and neoplasms were diagnosed according to the histological criteria of the Institute of Laboratory Animal Resources **(Stewart *et al*., 1980)** on the hematoxylin and eosin-stained sections.

3.13. Immunohistochemical staining of P glutathione-s-transferase

Localization of GST-P activity was carried out according to the protocol of **Tatematsu *et al*. (1988).** Paraffin embedded liver tissues were sectioned on positively charged slides and immunohistochemically stained according to kit manufacturer protocol (Dako, LSAB+system-HRP, North America, Inc.)

The primary antibody was Anti-GST-P polyclonal antibody prepared in rabbit (MBL Co., LTD, USA) whereas the secondary antibody was Polyvalent Biotinylated Antibody (Anti Rabbit antibody).

3.14. Image analysis

The mean area of hepatocyte altered foci and hepatic nodules in H & E stained sections as well as the mean area of enzyme altered foci (P_GST positive foci) were measured using image analyzer Leica Quin 500 (pathology department, NRC). The area percent of enzyme altered foci per microscopic field were measured in 10 microscopic fields and the mean value was calculated.

3.15. Statistical analysis

Statistical analysis were performed by the statistical package SPSS, version 8.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis of data was carried out using one-way analysis of variance (ANOVA) followed by LSD and Duncan test. Results were expressed as mean ± standard error (mean ± SE). *p* values less than 0.05 were considered significant and *P* less than 0.01 was considered highly significant.