

Effect of different beef sausage formulas on rat liver with focusing on their nitrosodiethylamine contentManar, M. A. Farag¹, Faten. F. Mohammed² and Amany, A. Salem¹¹Food Technology Research Institute, Agricultural Research Center, Ministry of Agriculture.²Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Giza, EgyptCorresponding author mail: fatenfathy21@yahoo.com, fatenfathy@staff.cu.edu.eg

Abstract: This study was performed to examine the possibility for alleviating the deleterious effect of nitrosodiethylamine (NDEA) that formed during processing of beef sausage by supplementation of a mixture of herbs and beetroot juice; for this purpose a total 36 adult male rats (120±5g) were randomly divided into 6 groups (G1) negative control group that fed on basal diet, (G2) positive control that treated daily with nitrosodiethylamines in drinking water at concentrate 40ppm_{NA} /100g_{b.w} for 5 weeks, (G3) rats treated with the previously described treatment protocol of G2 plus mixture of herbs and beetroot juice as protection, (G4) rats fed on standard sausage formula, (G5) rats fed on experimentally modified sausage and (G6) rats fed on commercial sausage (unknown composition). The duration of the experimental period was 19 weeks except the G3 that was 21 weeks. Estimation of NDEA in different beef sausage formulas, serum, and urinary excretion in addition to histopathological evaluations were performed. Results indicated that NDEA level was higher in commercial sausage formula compared with other sausage formulas as NDEA content in food, serum and urine were (0.61 µg/100gm, 18.10 µg/dl and 40.30 µg /dl, respectively), significantly decreased in experimentally modified sausage formula to be 0.111µg/100gm, 0.64µg/dl and 7.848µg/dl, respectively. Various histopathological alterations were detected in all treated groups that were more severe in positive control group, the lesions include appearance of altered hepatocellular foci (AHF) and bile duct and oval cell hyperplasia, these lesions were ameliorated in G3, while the worst histopathological lesions were recorded in rats group fed on commercial sausage compared with fed on other sausage formulas. **Conclusion:** The supplementation of herbal mixtures and beetroot juice in beef sausage formula able to reduce the deleterious effect of NDEA that formed during processing meat products.

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Key words: Nitrosodiethylamines (NDEA) - beef sausage formulas - herbs -beet root juice- serum - urine - histopathology.

1-Introduction:

Red meat is an important dietary source of protein, providing all essential amino acids and an excellent source of nutrients including iron, zinc, vitamin B₁₂ and conjugated linoleic acid (CLA), while its consumption may increase the risk of cardiovascular disease in addition **World Cancer Research Fund and American Institute of Cancer Research (2007)**, (WCRF/AICR) concluded that high intake of red and processed meat increases the risk of colorectal cancer. Nitrites or nitrates that were added to meat for preservation could increase the exogenous exposure to nitrosamines, N-nitroso compounds, and their precursors. N-nitrosodiethylamine known as nitrosodiethylamine(NDEA) is belonged to the family of carcinogenic N-nitroso compounds and considered as an environmental carcinogen and highly mutagenic [**Stemhagen et al., 1983**]. Nitrosamines are well known hepatic carcinogens and cause of liver necrosis (**Tricker and Kubacki, 1992**). The average human food intake of nitrosamines is 1 mg/day [**Sadik et al., 2008**] while **Aune et al. (2013)** suggested that the daily consumption of fresh red and processed meat must be

no more than 100g and 50g/day, respectively. Nitrosamines are produced from nitrites and secondary amines. The heat temperatures can enhance the formation of nitrosamines (**Jakszyn, and Gonzalez, 2006**). Nitrites give the meat the desirable cured color by combining with heme iron forming nitrosylmyoglobin. Cooking denatures globin which detaches from the heme, yielding a pink mononitrosylheme complex that gives the color of cooked cured meat (**Shahidi and Pegg, 1991**).

Haem-iron may facilitate the formation of nitroso-compounds in the absence of colonic flora in the upper human gastrointestinal tract, where Nitrosyl-haemoglobin, formed in acidic conditions equivalent to those in the stomach (**Alam et al., 2013 and Serban, 2013**). Also, Haem iron promotes carcinogenesis through increased cell proliferation in the mouse mucosa by inducing lipid oxidation. Thus the diet is unbalanced due to a high intake of meat, such protective mechanisms may not be sufficient to protect the colon from DNA damage [**Jssennagger et al. (2013)**]. The boiling and microwave treatments are considered as the most suitable methods for cured meat

Drabik-Markiewicz et al. (2009) and Drabik-Markiewicz et al. (2011).

Vegetables such as; celery, radishes, beets and spinach, have high levels of nitrate that can be metabolized into nitrite by oral bacteria (**Hord et al., 2009**).

In Central and Eastern Europe, red beetroot is a popular vegetable, known for a long time for its beneficial health effects: stimulation of hematopoietic and immune systems, hepato and renal protection in addition, it was considered as a special diet during cancer treatment. Protective effect of red beetroot is related to antioxidant, anti-inflammatory and antitumor properties (**Georgiev et al., 2010**). Besides diverse polyphenols, red beetroot contains betalains, a family of non-phenolic and water-soluble antioxidants which comprise red betacyanins and yellow betaxanthins (**Kanner et al., 2001**) and play a protective role against carcinogen induced oxidative stress in rats **Krajka-Kuzniak et al. (2012)**. The few reports published on the chemo preventive action of red beetroot which is considered as an important potential for this vegetable (**Platt et al., 2010**).

Spices and herbs are used in foods for their flavor and in medicinal mixtures for their physiological effects related to their high concentrations of phenolic compounds (**Muchuweti et al., 2007**). Where, the demand for natural bioactive compounds is increasing due to their use in the functional food. Rosemary, clove and cinnamon are the most appreciated natural sources for bioactive compounds. Cinnamon contains a number of anti oxidative components especially polyphenolic compound (**Muchuweti et al., 2007**). Rosemary is a popular medicinal herb and its extract (RE) has a significant anti proliferative activities against a variety of human cancer cell lines (**Yesil-Celiktas et al., 2010**). The main anti oxidative effect of rosemary has been related to the presence of three phenolic compounds carnosic acid, carnosol, and rosmarinic acid (**Cuvelier et al., 1996**). The primary components of clove (*Eugenia caryophyllus*) essential oil are phenylpropanoids (**Chaieb et al., 2007**). The antioxidant activity of clove is related to glycosidically bind volatile compounds content (**Politeo et al., 2010**). Heating at 100 °C for up to 6 h increases the peroxy radical-scavenging activity of clove (**Khatun et al., 2006**).

The aim of this experiment to determine the NDEA content in different sausage formulas and evaluate the protective effect of some herbal mixtures supplementation in sausage formulas against hepatic pathology induced by nitrosodiethylamine (NDEA) that was formed during processing of beef sausage.

2-Materials and Methods:

2-1-Materials:

1-Beef meat and fat tissues (sheep tail): Imported frozen beef meat from boneless round (Brazilian product) and fat tissues (sheep tail) were purchased from private shop in the local market at Giza, Egypt.

2-Other ingredients for processing sausage formulas such as; Extruded soy bean protein obtained from Food Technology Research Institute, Agriculture Research Center, Giza, Egypt. Sodium nitrite, tripolyphosphate (El-Gomhoria for chemicals Company, Egypt), the mixture of spices and herbs (Rosemary, clove and cinnamon) were obtained from the Horticultural Research Institute, Agricultural Research Centre, Giza, Egypt. beet root were obtained from local market at Giza, Egypt. Sodium nitrite, tripolyphosphate and, food salt (Sodium chloride) were obtained from El-Gomhoria for chemicals Company, Egypt and ascorbic acid (British Drug Houses LTD, Pool, England). Nitrosodiethylamine standard (NDEA) was obtained from Sigma-Aldrich, St. Louis, USA. Kits were obtained from Randox Laboratories Ltd., Diamond Road, Crumlin, Co., Antrim, United Kingdom, BT. 294QY.

2-2-Methods:

Technological methods:

1- Preparation of materials:

1- Beet root was washed well and peeled, adding water to peeled beet root (2:1v/w), grinded well in blender, pass throw cotton piece for removing fiber and must be fresh (daily work).

2- Spices and herbs were powdered in a laboratory mill and a mixture of powdered spices or spices and herbs were prepared for processing different beef sausage formulas.

2- Preparation of different sausage formulas:

Both beef meat and fat tissues (sheep tail) were grinded separately through 4.5mm plate (twice). All formulas were prepared by mixing minced lean beef with ice and salt for 5 min in a laboratory chopper (Hobart Kneading machine, Italy), then other ingredients were added and emulsified or chopped for another 5 min (adding beet root juice and herbs for tested sausage formula instead of nitrite which added to control sausage formula). Obtained emulsion were stuffed in natural mutton casings, then samples placed in fibrous plates, wrapped with polyethylene film and kept at -18C° for further analysis. Composition of different sausage formulas (g/100g) are shown in table (1).

Analytical methods:

Protein and fat of used sausage formulas (standard, experimentally modified and commercial) for preparing different diets were evaluated according to **A.O.A.C. (2005)**. Total carbohydrates were collected by difference.

Determination of nitrosodiethylamines (NDEA):

Determination of nitrosodiethylamines (NDEA) by using HPLC apparatus in foods samples (different beef sausage formulas) according to methods described by Kane, et al., (1990) and Sen and Seaman (1984) for serum and urine.

Biological evaluation of tested formulas:

a- Animals and experimental design:-

Thirty six adult male Sprague-Dawley albino rats average weight (80±5 g) were purchased from the Laboratory Animal Department, Research Institute of Ophthalmology, Giza, Egypt. The animals were housed in plastic cages under normal health laboratory condition at 21°C ± 2°C with timed lighting 12h and relative air humidity of 40 - 60% and fed on basal diet for one week as an adaptation period. After the adaptation period (1 week), the rats were randomly divided into 6 groups (6 rats for each group). The composition of the experimental diet for each group is shown in table (2) according to the method described by Association of Analytical Chemistry A.O.A.C. (2005).

Table (1): Composition of different sausage formulas (g/100g)

Ingredients	Standard sausage	Experimentally modified sausage*
Meat	60.0	60
Fat (sheep tail)	15.00	15.00
Cooled water	10.00	-
Beet root juice	-	10.00
Extruded soybean	8.70	8.70
Starch	2.00	2.00
Skim milk	1.69	1.69
Salt	1.50	1.50
Spices mixtures	1.10	1.10
Nitrite	0.0125	-
Total	100	100
Herbs	-	1.5

*1.5 % herbs consists of rosemary, clove and cinnamon, (1:1:1) was added to the tested sausage formula.

Table (2): Composition of the experimental diets (%)*

Items	Control (-)	Control (+)	Control (+)**	Standard sausage	Experimentally modified sausage	Commercial sausage
Casein (as protein)	17	17	17	--	--	--
Standard sausage	--	--	--	83	--	--
Experimentally modified sausage	--	--	--	--	83	--
Commercial sausage	--	--	--	--	--	90
Fat (corn oil)	10	10	10	-	--	-
Cellulose	5	5	5	5	5	5
Vitamins mix.	1	1	1	1	1	1
Salt mix.	4	4	4	4	4	4
Sucrose	21	21	21	2.3	2.3	--
Starch	42	42	40.5	4.7	4.7	--
Rosemary	--	--	0.5	--	--	--
Clove	--	--	0.5	--	--	--
Cinnamon	--	--	0.5	--	--	--
Total	100	100	100	100	100	100

* (AOAC, 2005) ** 1ml/day/rat of beet root juice.

The data explain beef sausage formulas weight as equal 15% protein, supplemented with 4% salt mixtures and 1% vitamin mixtures and completed to 100% with sucrose and starch where the last 2-fold the first.

The blood samples were collected each 15 days throughout and at the end of experimental period. The blood samples were collected from eye plexuses into both heparinized tubes and into a dry clean centrifuge glass tube without any coagulation to prepare serum. Blood samples were left for 15 minutes at room temperature, then the tubes were centrifuged for 15 min at 3000 rpm and the clean supernatant serum was kept

frozen at -20 °C until the time of analysis. At end of the experimental period rats were weighed and euthanized under deep anesthesia using ether, their livers were separated and collection of tissue specimen were performed for further histological examination.

c-Histopathological and immunohistochemical examination:-

Tissue specimens were collected from liver preserved in 10% neutral buffered formalin, dehydrated in different grades of alcohol, cleared in xylene, embedding in paraffin, sectioned with microtome at 5µ thickness and finally stained with hematoxylin and eosin (H&E) and Periodic acid Schiff's (PAS)

according to (Bancroft *et al.*, 1996) on other hand tissue section of blood vessels were dewaxed in xylene, rehydrated and pretreated with 3 % hydrogen peroxide for blocking the activity of endogenous peroxidase. microwave as sited antigen retrieval was done for 20 minutes and sections were incubated overnight at 40 °C with primary antibody for cytokeratin7 (CK7,RCK105] (ab9021)was dilutes with phosphate buffer saline (PBS) (1/1000.) then washed with PBS and incubated with biotinylated mouse secondary antibody (Cat No.32230,ThermoScientific Co., UK) and finally conjugated with streptavidin-peroxidase. Sections were washed with PBS and incubated with diaminobenzidid (DAB) for 5 minutes.

d-Statistical analysis:

Statistical analyses were carried out by SPSS10 program. Data were expressed as means \pm SEM and the

Statistical analysis was performed using one-way analysis of variance followed by Duncan's tests.

3-Results and Discussions:

Chemical composition of different beef sausage formulas:

Chemical composition of different beef sausage formulas samples are shown in table (3). The obtained results indicated that the moisture content of commercial sausage formula was lower than standard and experimentally modified sausage formulas reached to 53.9, 57.3 and 56.9 g/100g sample, respectively. The lower moisture content in commercial sausage may be related to presence of filling materials such as carbohydrates more than protein resulting in increase water holding capacity (W.H.C).

Table (3): Chemical composition of different sausage formulas (g/100g).

Formulas	Moisture	Protein	Fat	Ash	Carbohydrates
Standard sausage	57.3	18	17.2	4.0	3.5
Experimentally modified sausage	56.9	18	17.2	4.2	3.7
Commercial sausage	53.9	16	15.0	3.2	11.9

The previous data indicated that both protein and ash content were higher in both standard and experimentally modified sausage formulas than commercial one as protein and ash content in standard, experimentally modified and commercial sausage formulas reached to (18 and 4.0), (18 and 4.2) and (16 and 3.2) g/100g sample, respectively. The increased protein and ash content in both previously mention sausage formulas are due to their content of beef and

soybean and skim milk that were more than commercial sausage formula of unknown components.

Carbohydrates content in commercial sausage formula was 11.9g/100g sample higher than other sausage formulas due to add of filling materials instead of protein.

Concentration of nitrosodiethylamine (NDEA) in different beef sausage formulas:

Concentration of nitrosodiethylamine (NDEA) in different beef sausage formulas are shown in figure (1).

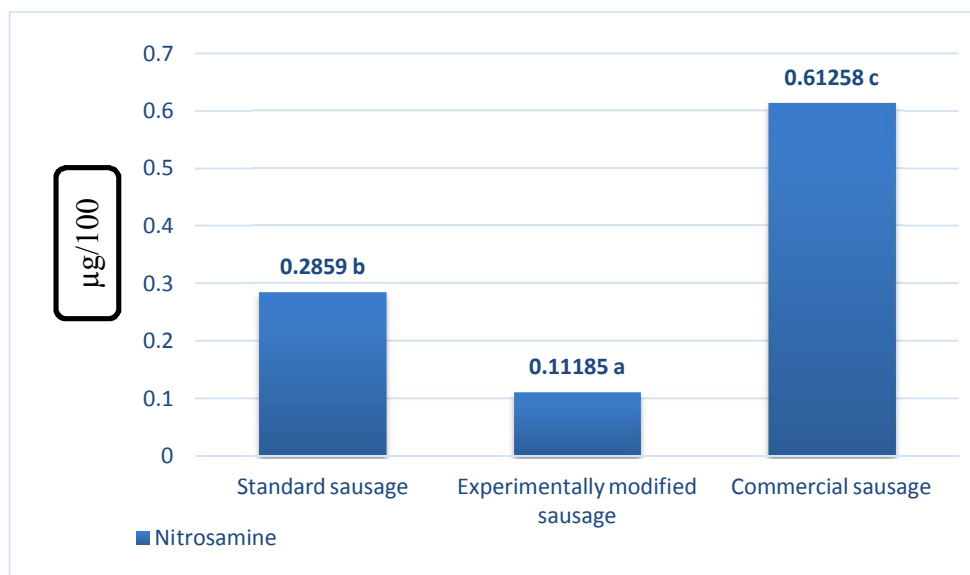


Fig (1): Nitrosodiethylamine (NDEA) concentration in standard, experimentally modified and commercial sausage formulas (µg/100g).

The results observed a significant differences of NDEA content among different treated groups which fed on different beef sausage formulas where the highest content was recorded in commercial sausage formula (their components unknown), followed by the standard sausage formula that contains 125ppb of sodium nitrite while the least value was recorded in experimentally fortified sausage formula which contains mixtures of herbs moreover plus the presence of beet root juice as natural source of nitrate instead of sodium nitrite and the reduction percent of NDEA reach approximately to 60 and 80% in experimentally modified sausage compared with standard and commercial sausage formulas, respectively. Also, the present results clarified that NDEA concentration in standard sausage formula decreased approximately by about 50% compared with commercial sausage formula.

The previous data was in agreement with **Katarzyna and kowalski (2003)** who observed that the level of total volatile N-nitrosamines was $3.15 \mu\text{g/kg meat}$ and **Li et al. (2012)** who found that the initial dry-cured raw sausage contained N-nitrosamines about $5.31 \mu\text{g/kg meats}$. Also, **Lunn, et al. (2006)** demonstrated that the apparent total N-nitroso compounds (ATNC) concentration in the diets was $22 \mu\text{g ATNC/kg}$ in raw meat (RM) and $37 \mu\text{g ATNC/kg}$ in processed meat (PM), and $9 \mu\text{g ATNC/kg}$ in the no

meat diet. In addition, **Campillo et al.(2011)** clarified that nitrosodiethylamines (NDEA) content was ranged from 1.9 to $3.6 \mu\text{g/kg}$ in dry-cured raw sausage supplemented with paprika. Previous researches clarified that boiling and microwave treatments were the most suitable methods for cured meat and the formation of N-nitrosamine occurred at high cooking temperature as done by deep-frying or pan-frying of meat products. Studies indicated that the levels of NA in processed meat may increase during further processing, frying, baking or other heat treatments; the nitrosamine content was $10 \mu\text{g/kg}$ in processed meat contained 120 mg nitrite (**Drabik-Markiewicz et al., 2009 and Drabik-Markiewicz et al., 2011**). Our results indicated that the NDEA level in standard and experimentally modified sausage formulas was within the permissible limits according to **World Cancer Research Fund and American Institute of Cancer Research (2007)** who permitted average daily intake of NDEA to be $2.0\text{--}4.0 \mu\text{g/kg}_{\text{b,w}}$ while the NDEA content in commercial sausage formulas is exceed the permissible limits

Nitrosodiethylamine (NDEA) concentration ($\mu\text{g/dl}$) in serum and urine of rats in different treated groups:

Comparative evaluation of NDEA content ($\mu\text{g/dl}$) in serum and urinary excretions among treated rat groups that fed on experimental diets are shown in table (4).

Table (4): Nitrosodiethylamine (NDEA) content in serum and urine of rats fed on the experimental diets ($\mu\text{g/dl}$).

Groups	Nitrosodiethylamine (NDEA) $\mu\text{g/dl}$	
	Serum	Urine
Control (-)	ND	ND
Control (+)	$18.480 \pm 0.159^{\text{d}}$	$25.255 \pm 0.107^{\text{c}}$
Control (+) **	$9.283 \pm 0.291^{\text{b}}$	$15.428 \pm 0.401^{\text{b}}$
Standard sausage	$14.760 \pm 0.329^{\text{c}}$	$27.736 \pm 0.574^{\text{d}}$
Experimentally modified sausage	$0.640 \pm 0.006^{\text{a}}$	$7.848 \pm 0.760^{\text{a}}$
Commercial sausage	$18.105 \pm 0.313^{\text{d}}$	$40.303 \pm 0.449^{\text{e}}$

ND: Not detected.

*Each value in a row followed by the same letter are not significantly different at ($p \leq 0.05$).

** Intake herbs with diet and 1ml beet root juice by stomach tube /rat / day.

The results showed a significant differences in NDEA content in serum and urine among different treated groups as there was a significant increase in NDEA content in both serum and urine of control positive and commercial sausage fed groups followed by standard sausage group fed while a significant reduction in NDEA content in serum and urine occurred in experimentally modified sausage fed group followed by group 3 that was treated with DENA in drinking water and was administrated a mixture of herbs and beet root juice as the NDEA reduction % in serum and urine for group 3 reached approximately to 50 and 40 % compared with control (+) group. On other hand the lowest value of NDEA content in serum and urine (0.64 and $7.85 \mu\text{g/dl}$ respectively) were

recorded in rats fed on the experimental sausage with the reduction percents for NDEA in serum and urine reached approximately about 95 and 70% compared with group fed on standard sausage and 95 and 80% compared with commercial sausage fed group while NDEA was not detected in serum and urine in control negative group which fed on basal diet. These results are agreed with **Yamamoto et al.(1980)** who estimate the nitrosodiethylamine (NDEA) level in 32 serum samples from eight donors and they found the level was ranging from 0.5 to 1.3 ng/ml .

The results of body and liver weight of rats fed on different experimental diets are shown in table (5).

Results showed there was significant increase in total body and liver weight in group of rats fed on

commercial sausage formulas compared with the control and this may be due to adding of fillers as rich source of carbohydrates and increased fat content led to fatty change of the liver and increase the liver weight and final body weight on other hand a significant reduction in body and liver weight was achieved in control positive group that was related to the toxic injury of NDEA that alter body organ system function similar finding was recorded by **Masahiko Kushida et al. (2011)** whereas the reduction in body weight induced by DENA is ameliorated by adding herbal mixture plus beet root juice on other hand, there is no significant difference in body weight or liver weight of standard sausage fed group compared with the control while the body weight was increased significantly in experimentally modified sausage fed group compared

with the control but no significant difference in liver weight of this group compared with the control one this confirm the mild histopathological hepatic alterations that observed and prove the protective effect of the herbal mixture plus beet root juice that either added to the water contained NDEA in group 3 or to the sausage formula in experimentally modified sausage fed group. The previous obtained results in the present work agreed with **Krajka-Kuz'niak et al. (2012)** who clarified that the beetroot juice reduced the DNA damage induced by NDEA treatment, as well as improved liver biomarkers as it was considered as rich source of a specific class of antioxidants, betalains thus had a protective role against carcinogen induced oxidative stress in rats.

Table (5): Body and Liver Weights in different treated groups

Groups	Initial body weight	Final body weight	Body weight gain (g)	Body weight gain (%)	Liver weight (g)	Relative liver weight (%)
Control (-)	122.33±3.33 ^a	199.67±9.17 ^c	77.34±3.89 ^{bc}	63.22	6.27±0.30 ^b	3.14±0.11 ^a
Control (+)	122.34±3.71 ^a	177.68±6.69 ^d	55.34±7.75 ^c	45.23	4.86±0.10 ^c	2.71±0.06 ^{bc}
Control (+)**	121.67±2.80 ^a	197.33±6.57 ^{cd}	75.67±3.39 ^{bc}	62.18	5.97±0.12 ^b	3.03±0.14 ^{ab}
Standard sausage	120.32±3.67 ^a	198.32±6.44 ^c	78.00±5.57 ^{bc}	64.83	5.63±0.07 ^b	2.85±0.12 ^{abc}
Experimentally modified sausage	120.66±4.09 ^a	220.66±3.71 ^b	100.00±5.557 ^{ab}	82.88	5.73±0.28 ^b	2.60±0.15 ^c
Commercial sausage	120.00±3.41 ^a	245.34±4.37 ^a	125.34±6.52 ^a	104.45	7.20±0.32 ^a	2.94±0.13 ^{abc}

* Each value in a column followed by the same letter is not significantly different at ($p \geq 0.05$).

** Intake herbs with diet and 1ml beet root juice by stomach tube /rat / day.

Histopathological findings:-

Lesions in the NDEA treated group were illustrated in figure (1). The microscopic examination of the liver of NDEA treated group revealed presence of multiple altered hepatocellular foci (AHF) of varying size and type. The most commonly observed AHF was the clear cell foci that consisted of relatively larger hepatocytes with clear vacuolated cytoplasm, this foci was stained positively with PAS. the second type was the eosinophilic cell foci that consisted of hepatocytes with pale eosinophilic cytoplasm and containing individual altered clear hepatocytes, previous studies demonstrated the induction of AHF by certain chemical treatment and the incidence, size, and/or multiplicity of foci were usually increased after administration of hepatocarcinogens specifically treatment with nitrosamines (**Frith et al., 1994 and Moore et al., 1996**), occasional mitosis was detected in individual hepatocytes, polyploidy that represented by hepatocytomegally, karyomegally and anisokaryosis was evident these changes may be due to NDEA treatment as discussed by **Harada et al. (1996)** who found that Polyploidy increased with age in some strains of mice as well as following some treatment regimens resulting in hepatocytomegaly as well as karyomegaly. In the present study foci of proliferating

oval cells that showing mild nuclear atypia in the form of increased nuclear size with mild pleomorphism, **Bob Thoolen et al. (2010)** demonstrated that oval cell hyperplasia could be observed following severe hepatotoxic injury and treatment with hepatocarcinogens. Lesions in the group that was treated with NDEA plus mixture of herbs and beetroot juice were illustrated in figure (2), the previously described hepatic alterations were evident but with decreased lesion grade as the number of AHF were markedly decreased and of clear cell type only that showing necrosis of individual altered cells in addition to apoptosis, bile ductular hyperplasia and oval cell proliferation with formation of small cluster of regenerative small hepatocytes. These previously described results confirm the protective effect of the herbal treatment against the deleterious action of NDEA of hepatocytes in addition apoptosis was enhanced by herbal treatment this indicating the alleviating action of herbs against the inhibitory action of NDEA against apoptosis as Inhibition of apoptosis had a key role in the process of carcinogenesis (**Foster, 2000**). lesions in rats fed on different diet sausage formulas were illustrated in figure 3. In commercial sausage fed group, the liver sections showing multifocal microvesicular steatosis with increased

number of binucleated hepatocytes and the most obvious hepatic alteration was marked focal bile ductular hyperplasia with frequent mitosis of biliary epithelium. Oval cell hyperplasia was the most prominent hepatic alteration in standard sausage fed group with bile duct hyperplasia and apparently histological hepatocellular morphology except for individual hepatocellular necrosis while in experimentally modified sausage fed group the lesions

were mild and restricted to mild oval cell proliferation. The previous improvement of histopathological picture in experimentally treated sausage fed group was presumably related to antioxidant action of the supplemented herbal mixture plus beetroot juice as confirmed by **Krajka-Kuz'niak *et al.* (2012)** as they demonstrated that beetroot juice had the ability to reduce the DNA damage and of liver injury induced by NDEA treatment.

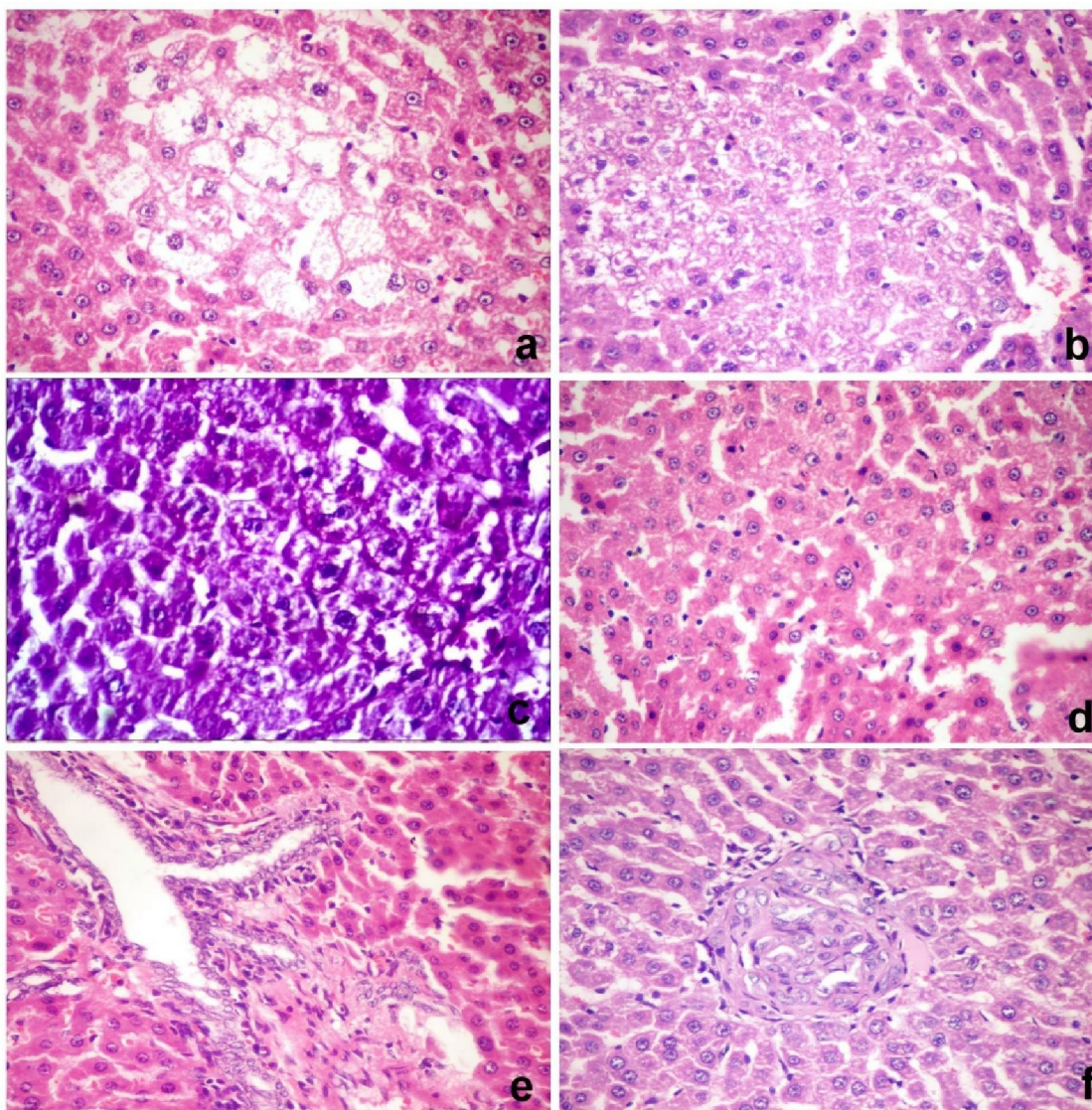


Fig.1): liver of rat treated with NDEA in drinking water : a) altered clear hepatocellular foci with hepatocytomegally and vacuolization of cytoplasm (H&E ,X400).b) Altered eosinophilic hepatocellular foci characterized by enlarged hepatocytes and abundant pale eosinophilic cytoplasm (H&E ,X400).c) PAS positive stained altered hepatocellular foci(PAS,X400).d) hepatocytes showing hepatocytomegally ,karyomegally and anisokaryosis (H&E,X400) .e)portal area showing bile duct hyperplasia (H&E,X400).f) small foci of oval cell proliferation that showing nuclear atypia (H&E,X400).

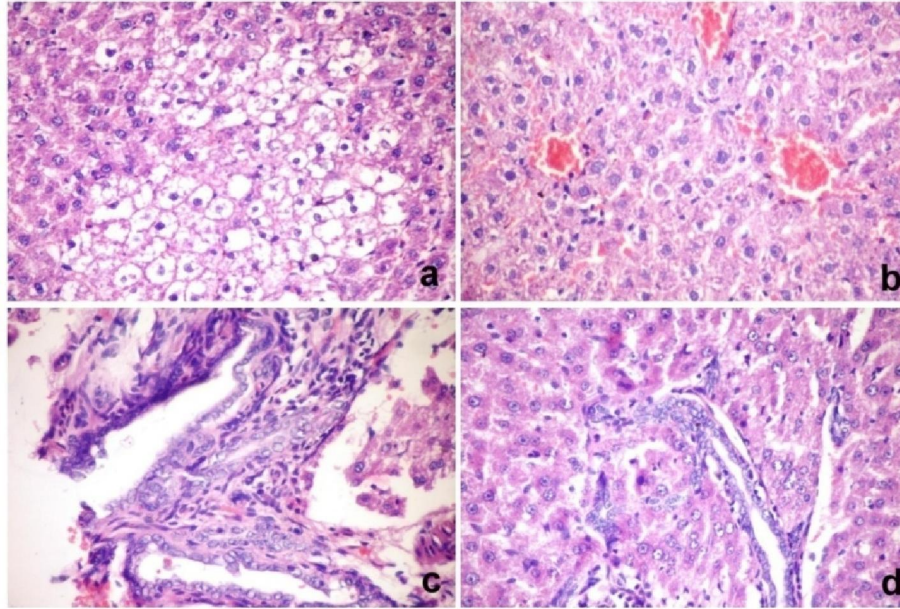


Fig.2): liver of rat treated with NDEA in drinking water plus herbs and beetroot juice (H&E stained histological sections): a) altered clear hepatocellular foci with nuclear pyknosis of altered hepatocytes (X400).b) hepatocytes showing apoptosis of individual hepatocytes(X400).c) portal area showing bile duct hyperplasia with newly formed bile ductules (X400).d) liver showing oval cell proliferation with formation of new small sized regenerative hepatocytes (X400).

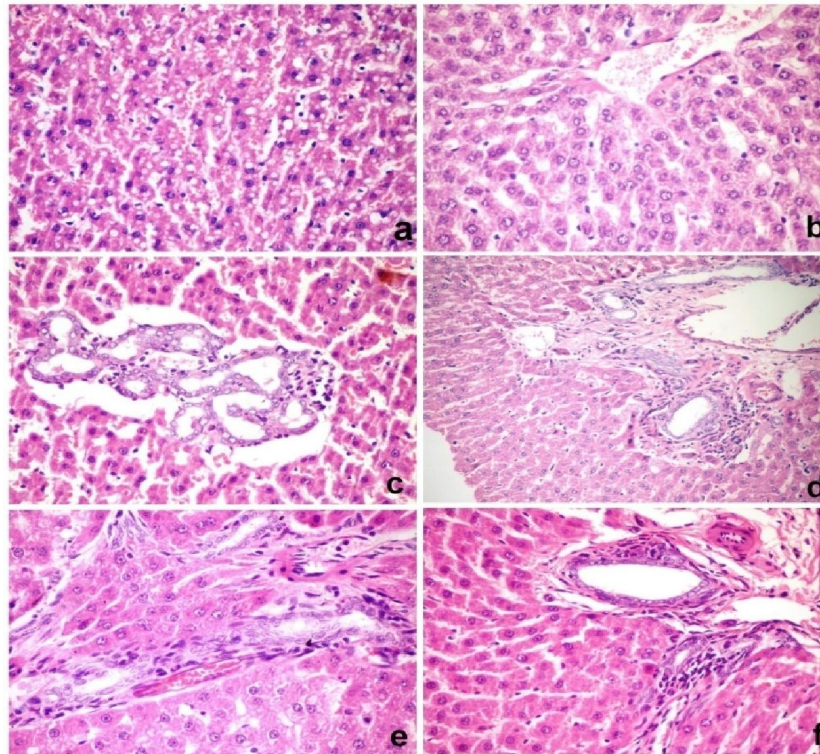


Fig.3): liver of rat fed on different sausage formula (H&E stained histological sections): a) commercial sausage fed group showing area of microvesicular steatosis (X400).b) commercial sausage fed group showing increased number of binucleated hepatocytes. (X400).c) commercial sausage fed group showing focal bile ductular hyperplasia with active vesicular nuclei and mitosis(X400).d) standard sausage fed group showing oval cell proliferation with mild bile duct p hyperplasia and few mononuclear cell infiltration (X200).e) standard sausage fed group showing oval cell proliferation extending from portal area to the adjacent hepatic parenchyma (X400).f) experimentally modified sausage fed group showing mild oval cell and bile duct hyperplasia with relatively little mononuclear cell infiltrating the portal area (X400).

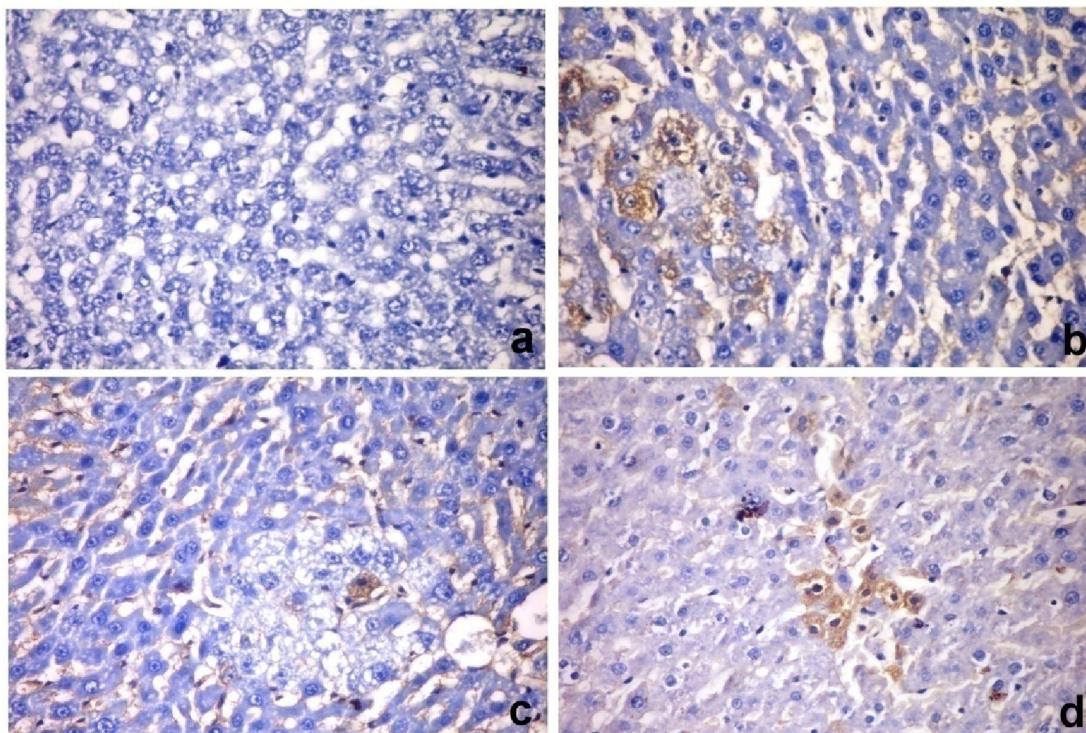


Fig.4): liver of rat of different treated groups (cytokeratin 7(CK7) immunohistochemistry stained sections): a) control negative untreated group showing negative staining reaction of hepatocytes for CK7 (X400).b) NDEA treated group showing moderate positive CK7 expression of most hepatocytes in the altered hepatocellular foci (X400).c) NDEA plus herbs and beetroot juice treated group showing expression of CK7 in individual hepatocytes (X400).d) commercial sausage fed group showing moderate positive CK7 expression of few numbers of hepatocytes (X400).

Immunohistochemistry:-

The histological evaluation of hepatic sections revealed abnormal expression of cytokeratin 7 in individual hepatocytes in NDEA, NDEA plus Protection and commercial sausage fed groups and was negative in the remained groups, the staining was more evident in NDEA treated rats and commercial sausage fed group in comparison to NDEA plus herbal and juice supplementation treated group(Fig.4). Previous researches clarified that CK7 was strongly expressed by interlobular bile ducts, intraportal and intralobular bile ductules and the biliary epithelial cells that partly line the canals of Herring but not expressed in hepatocytes (**Bateman and Hubscher 2010**) while **Eleazar, Memeo et al. (2004); Fotiadu, Tzioufa et al. (2004)** demonstrated that in sever hepatic disease some hepatocytes can express CK7 and the increased expression was parallel with the severity of hepatic disease grade and stage, also **Chaoling Ren et al. (2003)** proved that Cytokeratin 7 staining of hepatocytes predicted with a high degree of sensitivity and specificity progression to more severe liver disease in alcohol-fed baboons.

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