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Effect of Distillery Vinasse on the Productive Performance, DNA Damage, Expression of IGFBPs Gene and Histopathological Changes in Japanese Quail Fed Diets Contaminated with Phenol

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Abstract: The objectives of the present study were to evaluate the efficacy of vinasse (a molasses fermentation by-product) to ameliorate the productive performance, DNA damage, modulation in the expression of Insulin-like growth factor binding proteins (IGFBPs) gene and the histopathological changes in Japanese quail fed diets contaminated with phenol. Eighty (3 weeks old) male Japanese quail were randomly divided into eight groups, seven treatments and one control. Control group was fed a commercial quail starter diet (26% protein and 3150 ME Kcal/Kg). Four treatment diets were supplemented with vinasse (2.5%), vinasse (5%), vinasse (7.5%) and phenol (0.75gm/Kg diet). The other three treatments were the phenol contaminated diet with the three levels of vinasse. The quail groups received their prospective diets for 3 weeks. Compared to the control group, phenol significantly reduced body weight gain (BWG) as well as liver and testis weights. Also, phenol caused significant increase in the rate of DNA fragmentation, as well as significant down regulation of the expression level of IGFBPs gene and severe damage to liver, testis and breast muscle tissues. Addition of vinasse to the phenol contaminated diets significantly diminished most of the negative effects of the phenol. Vinasse ameliorated BWG, liver and testis weights, DNA damage, expression level of IGFBPs gene and the histopathological changes to different tissues. The improvements of such measurements were more pronounced in phenol plus vinasse (7.5%) diet than any of the other vinasse treatments. Moreover, the supplementation of vinasse, especially at the level of 7.5%, to the basal diet without phenol treatment, enhanced BW, BWG, liver and testis weights and the expression level of IGFBPs gene in the liver, testis and breast muscles tissues. These enhancements were significant in BW, BWG and the expression level of IGFBPs gene in liver tissue. However, there were no significant differences between control and vinasse treatments for the frequencies of DNA fragmentation. Also, histological results were relatively similar in each of the control and vinasse groups. These results indicated normal structure in the liver, testis and breast muscle tissues of the vinasse treated groups. In conclusion, our results proved the protective effects of vinasse against the harmful effects of phenol in quail feed. These positive effects of vinasse (7.5%) supplementation in quail rations, especially in case of phenol contamination suspicion, suppressed the toxic effects of phenol, reduced DNA damage, improved IGFBPs gene expression, ameliorated histopathological changes of several organs and consequently enhanced the productive performances of the birds.

Key words: Phenol · Vinasse · Quail · Productive Performance · DNA Damage · RT-PCR, Histopathology

INTRODUCTION

Phenols of anthropogenic origin exist in the environment due to the activity of the chemical, petrol and pharmaceutical industries [1]. Moreover, the occurrence of phenols in the environment is related to production and degradation of numerous pesticides and disinfectants [1, 2]. Also, some of these compounds are formed as a

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result of natural processes, especially during decomposition of organic matter or synthesis of chlorinated phenols by fungi and plants [1, 3]. Without proper treatment, these phenols will induce toxic activity and cause negative effects on the environment, since phenols are defined as one of the priority pollutants and is not easily degradable [4-6].

The toxic action of these compounds is related to hydrophobocity of the individual compound and formation of free radicals [1, 7]. Hydrophobocity affects the solubility of phenol in cells fractions and thus possibility of interaction of the compound with specified cell and tissue structures [1, 8]. Phenols, after perpetration of the cell, undergo active transformation, mainly at the participation of oxidases within cytochrome P450. Sometimes transformation processes lead to increase toxicity of individual compounds by the formation of electrophilic metabolites and lipid peroxidation that may bind and damage DNA or enzymes [1].

Phenols expressed their toxicity by forming a reactive oxygen species (ROS) that include a hydroxy radical that can interact with cellular DNA to form mutation leading to anomalies in DNA, genes and chromosomes as a result of disturbance of DNA replication [1]. ROS can also overwhelm antioxidant defense (protective enzymes) like superoxide dismutase, catalase and peroxidase and can cause destructive and lethal cellular effects (e.g. apoptosis) by oxidizing membrane lipids, cellular proteins, DNA and enzymes [1, 9, 10]. Also, several studies revealed that the phenol is accumulated in the liver, kidney, brain, lungs and also urogenital tract causing histopathological changes. Described changes are mainly induced by lipid peroxidation that is responsible for damage and finally degradation of cell's membrane [1, 11- 13].

Oxidative processes due to phenol toxicity may be inhibited by antioxidant agents. Artificial antioxidants such as butaleted hydroxyanisole (BHA), butaleted hydroxytoluene (BHT) and n-propylgallate exhibit strong antioxidant activity against several oxidation systems. However, because artificial antioxidants pose potential risks, *in vivo*, their use is restricted or prohibited in some countries [14]. Safer antioxidants from natural sources have therefore been investigated. A successful natural antioxidants or antimutagenes must be economically capable of eliminating most or all traces of toxins or dangerous metabolites without having harmful residues and must not impair the nutritional quality of the commodity [15, 16].

One approach of many to this problem is the use of vinasse for minimizing the adverse effects of phenols, in poultry, on the basis of biological protection [17-19]. Vinasse which is a by-product of industrial production of alcohol, or other substances by the fermentation of molasses [20, 21] has been identified to possess yeasts, polysaccharides, minerals, vitamins, nonessential amino acids, predominantly glutamic and aspartic acids [19, 21]. These compounds were found to be strong antioxidants and have potent free radical-scavenging activities, as well as they have antimutagenic effects against several mutagenes [22-28].

The uses of vinasse as a feed additive in poultry and pigs were reported by Stemme *et al.* [18] to show its influence on animal performance. The positive effects of adding vinasse are due to its contents of yeast walls (Polysaccharides, beta-glucans and manar type) minerals and B-complex vitamins [29, 30]. These compounds which were found to increase the efficiency of utilization of nutrients, can exert effects on the immune system of the chicken, exclusion of pathogens at digestive scale and therefore, produces birds with a better performance.

Proper feeding of birds begins in the first weeks of life and must be followed strictly during the period of growth. This is fundamental for achieving maximum efficient meat and egg production [31]. The measurement of activity of Insulin-like growth factor binding proteins (IGFBPs) in such stage of life is considered to be a good indicator for growth promoters [32]. Insulin-like growth factors (IGFs) play critical roles in proliferation, differentiation and transformation in a variety of vertebrate tissues [33]. It has been thought that IGF-I is produced in the liver, secreted into the circulation and acts on target tissues in an endocrine manner. Besides its endocrine effects, IGF-I is also produced in most extrahepatic tissues and can function as an autocrine and/or paracrine growth stimulator [32, 33]. The actions of IGFs are modulated by IGF- binding proteins (IGFBPs). IGFBPs bind specifically and with high affinity to IGFs and they have been suggested to act as modulators by either enhancing or inhibiting the activity and bioavailability of IGFs [32, 33].

Thus, the present study was designed to evaluate the efficacy of vinasse to ameliorate body weight (BW), body weight gain (BWG), liver weight, testes weight, DNA damage, modulation in the expression level of insulin-like growth factor binding proteins (IGFBPs) gene and the histopathological changes in Japanese quail fed diets contaminated with phenol.

MATERIALS AND METHODS

Birds: Eighty unvaccinated 21 days old male Japanese quail chicks were obtained from the quail project, Faculty of Agriculture, Cairo University. The quail were divided into eight groups. Each group, of 10 quail, were weighted and placed in a heated wooden brooder (battery cage). The birds received continuous light for the duration of the experiment. The quail also received *ad lib*.water and a commercial growing quail ration (basal diet) containing 26% protein and 3150 Kcal ME/Kg diet. The diet also contained all the required amino acids, vitamins and minerals according to the recommendations of the National Research Council (NRC), [34] without adding antibiotics, coccidiostats or growth promoters. All birds received humane treatment in compliance with the guidelines of the Animal Care and Use Committee of the Faculty of Agriculture, Cairo University, Egypt.

Phenol Preparation: Phenol was purchased from the Water Pollution Department, National Research Center, Egypt. Twenty four grams of pure crystalline phenol were dissolved in 1L of sterile distilled water. This solution was then added to 32 Kg basal diet and homogenized to obtain the required final level of phenol which was 0.75gm/Kg diet [1, 35]. This dose of phenol has been considered a toxic experimental dose that affect birds organs [1].

Vinasse Diets Preparation: Distillery vinasse was obtained from the Sugar and Integrated Industries Company, Hawamdyia, Egypt. The Microbial Chemistry Department, National Research Center, Egypt, supplied by-product at three levels, 2.5% (200ml/8kg of diet), 5% (400ml/8kg of diet) and 7.5% (600ml/8kg of diet). These rates of vinasse were added and mixed well to the feed Hidalgo *et al*. [19]. The phenol was incorporated into the mixed feed before vinasse was added.

Experimental Design: Eight groups of quail were supplemented with eight dietary treatments as follows; (1) control, basal diet; (2) basal diet plus 2.5% vinasse/Kg diet; (3) basal diet plus 5% vinasse/Kg diet; (4) basal diet plus 7.5% vinasse/Kg diet; (5) basal diet plus 0.75gm phenol/Kg diet; (6) basal diet plus 0.75gm phenol/Kg diet, plus 2.5% vinasse/Kg diet; (7) basal diet plus 0.75 gm phenol/Kg diet plus 5% vinasse/Kg diet; (8) basal diet plus 0.75gm phenol/Kg diet plus 7.5% vinasse/Kg diet.

Performance Parameters: The trial period was carried out for 3 weeks. During the experiment, the birds were weighed weekly to determine their body weight (BW) and body weight gain (BWG) at 21, 28, 35 and 42 days of age, after fasting for 8 hrs, to the nearest gram using a digital scale. The cumulative body weight gains were calculated by subtracting W2 – W1.

Sampling Schedule: After the end of the experiment, all birds were slaughtered. Liver, breast muscle and testes were collected. Liver and testes were weighed and used with breast muscle to evaluate the expression levels of IGFBP gene as well as for histopathological examination. The rate of DNA fragmentation was assessed in liver tissues.

DNA Fragmentation Analysis: Liver tissues of quail were used to determine the quantitative profile of the DNA fragmentation using Diphenylamine reaction procedure. Briefly, liver samples were collected immediately after sacrificing the animals. The tissues were lysed in 0.5 ml of lysis buffer containing; 10 mM tris-HCl (pH 8), 1 mM EDTA, 0.2% triton X-100 then it was centrifuged at 10 000 r.p.m. (Eppendorf tubes) for 20 min at 4°C. The pellets were resuspended in 0.5 ml of lysis buffer. Half ml of 25% trichloroacetic acid (TCA) was added to the pellets (P) and the supernatants (S) and incubated at 4°C for 24 hrs. The samples were centrifuged for 20 min at 10,000 r.p.m. (Eppendorf tubes) at 4°C and the pellets were suspended in 80 ml of 5% TCA, followed by incubation at 83°C for 20 min. Subsequently, 160 ml of DPA solution [150 mg DPA in 10 ml glacial acetic acid, 150 ml of sulfuric acid and 50 ml acetaldehyde (16 mg:ml)] were added to each sample and incubated at room temperature for 24 hrs [36]. The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$\% \text{ Fragmented DNA} = \frac{OD(S)}{OD(S)+OD(P)} \times 100$$

Gene Expression Assay

Semi-Quantitative RT-PCR

Extraction of Total RNA: Liver, testis and breast muscles tissues of quail, from all groups, were used to extract total RNA using TRIzol® Reagent. Total RNA was treated with 1 U of RQ1 RNAse-free DNAse to digest DNA residues, re-suspended in DEPC-treated water and photo spectro metrically quantified at A_{260}. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1).

Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT), otherwise stored at -80°C.

Synthesis of the cDNA Using Reverse Transcription (RT) Reaction: The complete Poly $(A)^+$ RNA isolated from quail tissues was reverse transcribed into cDNA in a total volume of 20µl using RevertAid™ First Strand cDNA Synthesis Kit. An amount of total RNA (5µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3), 10mM of each dNTP, 50µM oligo-dT primer, 20U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The mixture of each sample was centrifuged for 30 sec at 1000 g and transferred to the thermocycler. The RT reaction was carried out at 25°C for 10 min, followed by 1h at 42°C and finished with a denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative RT-PCR.

Quantitative RT- Polymerase Chain Reaction (PCR): The first strand cDNA from different quail samples was used as templates for RT-PCR with a pair of specific primers. The reaction mixture for RT-PCR was consisted of 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltsd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0°C for 3 min. Second step consisted of 40 cycles in which each cycle was divided to 3 sub-steps: (a) at 95.0°C for 15 sec; (b) at 55.0°C for 30 sec; and (c) at 72.0°C for 30 sec. The third step consisted of 71 cycles which started at 60.0°C and then increased about 0.5°C every 10 sec up to 95.0°C. The semi quantitative values of RT-PCR (sqRT-PCR) of IGFBP-5 [37] 5'-TGC GAG CTG GTG AAG GAG CC-3' (sense) and 5'-TCA CTC CAC GTT GCT GCT GTC-3' (antisense), gene was normalized on the bases of β-actin (β-actin-F: 5'-CAC GTG GGC CGC TCT AGG CAC CAA -3', β-actin-R: 5'- CTC TTT GAT GTC ACG CAC GAT TTC-3' expression [38]. At the end of each sqRT-PCR a melting curve analysis was performed at 95.0°C to check the quality of the used primers.

The relative quantification of the target to the reference was determined by using the ΔCT method if E for the target (IGFBP) and the reference primers (β-Actin) are the same.

$$\text{Ratio}_{(\text{reference/ target gene})} = \text{Ef}^{C_T(\text{reference}) - C_T(\text{target})}$$

Histopathological Examination: Specimens of liver, testes and breast muscles from all animals were dissected immediately after death and fixed in 10% neutral-buffered formal saline for 72 hours at least. All the specimens were washed in tap water for half an hour and then dehydrated in ascending grades of alcohol (70% - 80% - 90% and finally absolute alcohol), cleared in xylene, impregnated in soft paraffin wax at 55°C and embedded in hard parrafin. Serial sections of 6 µm thick were cut and stained with Haematoxylin and eosin [39] for histopathological investigation. Images were captured and processed using Adobe Photoshop version 8.

Statistical Analyses: All data including DNA fragmentation analysis and gene expression assay were analyzed using the General Liner Models (GLM) procedure of the Statistical Analysis System [40] followed by Scheffe-test to assess significant difference between groups if differences exist. All statements of significance were based on probability of ($P \leq 0.05$).

RESULTS

Body Weight: Results in Table (1) showed the effect of dietary treatment on body weigh (BW). Feeding the phenol contaminated diet alone suppressed the BW from 5th week (BW5) to 6th week (BW6) as compared to the vinasse (7.5%) alone but not from the controls. This deleterious effect of phenol on BW was significant. The supplementation of vinasse at levels 2.5%, 5% and 7.5% to phenol-containing diets did not improve the adverse effect of phenol on BW during the 5th and 6th week of age (Table 1).

The supplementation of vinasse (2.5-7.5%) to the basal diet, without phenol contamination, increased the body weight from the 5th week until the end of the 6th week of age as compared to phenol contaminated diet.

Body Weight Gain (BWG): Feeding phenol contaminated diet, alone, also suppressed the BWG significantly (Table 2) from 3 weeks until the end of the experimental period as compared to the controls. This suppression in

Table 1: Effect of different treatments on quail body weight at different ages

Treatment	Initial body weight (3 weeks of age)	4 weeks of age (BW4)	5 weeks of age (BW5)	6 weeks of age (BW6)
Negative control	120.90±2.95	174.25±3.37	214.64±4.95^{ab}	237.45±6.01^{bc}
Vinasse (2.5%)	127.37±4.87	184.29±5.96	220.61±5.26^{ab}	242.99±4.13^{bc}
Vinasse (5%)	128.54±6.98	180.76±7.63	214.44±7.78^{ab}	248.21±5.62^{b}
Vinasse (7.5%)	134.51±3.46	188.57±4.24	232.85±5.97^{a}	265.78±4.28^{a}
Phenol	129.11±2.93	176.06±5.59	211.15±3.04^{b}	230.26±2.15^{c}
Phenol+Vinasse(2.5%)	126.65±3.36	173.14±6.70	215.08±6.71^{ab}	235.18±6.09^{bc}
Phenol+Vinasse (5%)	122.46±4.64	170.53±6.44	216.15±4.23^{ab}	238.81±3.91^{bc}
Phenol+Vinasse(7.5%)	121.08±2.93	173.71±3.79	217.03±5.04^{ab}	240.54±3.20^{bc}

Data are expressed as Mean ± SEM.

a, b and c means followed different superscripts are significantly different ($P \leq 0.05$).

Table 2: Effect of different treatments on quail body weight gain at different ages

Treatment	From 3-4 weeks of age	From 4-5 weeks of age	From 5-6 weeks of age	From 3-6 weeks of age
Negative control	53.35±1.98^{ab}	40.39±2.15^{ab}	22.81±2.58^{b}	116.55±5.07^{b}
Vinasse (2.5%)	56.92±2.63^{a}	36.31±2.34^{ab}	22.38±2.24^{b}	115.62±3.10^{b}
Vinasse (5%)	52.22±2.84^{ab}	33.68±4.15^{b}	33.77±3.20^{a}	119.67±6.40^{ab}
Vinasse (7.5%)	54.06±1.42^{b}	44.28±2.18^{ab}	32.93±3.28^{a}	131.27±3.09^{a}
Phenol	46.96±4.04^{b}	35.10±3.45^{ab}	19.11±2.72^{b}	101.15±3.03^{c}
Phenol+Vinasse(2.5%)	46.50±4.51^{b}	41.93±3.98^{ab}	20.11±1.95^{b}	108.54±5.29^{c}
Phenol+Vinasse (5%)	48.07±2.88^{ab}	45.63±3.32^{a}	22.66±3.26^{b}	116.35±4.41^{b}
Phenol+Vinasse(7.5%)	52.63±2.25^{ab}	43.32±3.14^{ab}	23.50±3.87^{b}	119.45±3.81^{ab}

Data are expressed as Mean ± SEM.

a, b and c means, within age, followed by different superscripts are significantly different ($P \leq 0.05$).

Table 3: Effect of different treatments on quail liver and testes weights at 6 weeks of age

Treatment	Liver weight	Testes weight
Negative control	5.17±0.26^{a}	5.23±0.26^{a}
Vinasse (2.5%)	5.37±0.23^{a}	5.37±0.80^{a}
Vinasse (5%)	5.40±0.26^{a}	5.59±0.45^{a}
Vinasse (7.5%)	5.41±0.13^{a}	6.42±0.92^{a}
Phenol	3.89±0.34^{b}	3.78±0.34^{b}
Phenol+Vinasse (2.5%)	4.42±0.23^{b}	4.39±0.54^{b}
Phenol+Vinasse (5%)	4.84±0.18^{ab}	4.56±0.78^{ab}
Phenol+Vinasse(7.5%)	5.19±0.20^{a}	4.91±0.46^{a}

Data are expressed as Mean ± SEM.

a, b and c means within organs, followed by superscripts are significantly different ($P \leq 0.05$).

Table 4: Rates of DNA fragmentation in liver tissues of quail fed different diets

Treatments	% DNA Fragmentation
Negative control	8.2+0.6^{b}
Vinasse (2.5%)	7.9+0.5^{b}
Vinasse (5%)	8.5+0.4^{b}
Vinasse (7.5%)	8.2+0.7^{b}
Phenol	28.3+0.7^{a}
Phenol+Vinasse (2.5%)	26.3+0.5^{a}
Phenol+Vinasse (5%)	21.3+0.5^{ab}
Phenol+Vinasse (7.5%)	13.7+0.5^{b}

Data are expressed as Mean ± SEM.

a and b means followed by different superscripts are significantly different ($P \leq 0.05$).

BWG was significant ($P \leq 0.05$) from the 3rd to the 6th weeks of age. The addition of vinasse (2.5%, 5% and 7.5%) to the phenol contaminated diets ameliorated the adverse effect of phenol on BWG. These enhancements were significant in quail groups fed diets contaminated with phenol plus vinasse at levels of 5% or 7.5% from the 3rd to 6th weeks of age. On the other hand, the addition of vinasse especially at 5% or 7.5% levels to the basal diet, without phenol contamination, increased the BWG from 5-6 weeks and from 3-6 weeks of age as compared to the negative control. These increases were significant ($P<0.05$) in quail fed basal diet plus vinasse at 5% or 7.5% level from 5-6 weeks of age. They were also significant ($P<0.05$) in quail fed the basal diet plus vinasse at 7.5% level from 3-6 weeks of age.

Effect of Different Treatments on Quail Liver and Testes Weights at 6 Weeks of Age: Data presented in Table (3) revealed that feeding phenol contaminated diet alone significantly ($P \leq 0.05$) decreased the liver weight at 6th week of age as compared to the control. The addition of vinasse at 2.5%, 5% and 7.5% levels to phenol contaminated diets ameliorated the deleterious effect of phenol on liver weight. This amelioration was significant ($P \leq 0.05$) in quail fed the diet contaminated with phenol plus vinasse at 7.5% level. Also, the results showed that

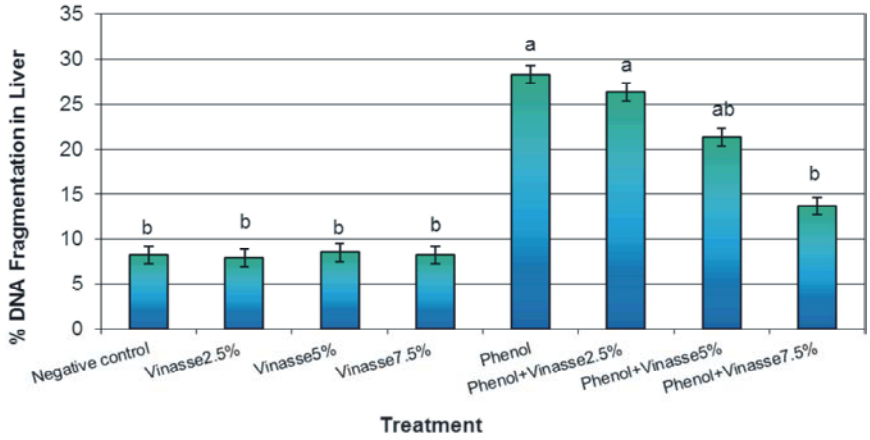

Fig. 1: DNA fragmentation rate in the liver tissue of quail fed diets contaminated with phenol and vinasse
Means with different letters are significantly different (P≤ 0.05)

the addition of vinasse at 2.5%, 5% and 7.5% levels to the basal diet increased the liver weight as compared to the negative control, however, these increases were not significant.

Table (3) showed that feeding phenol contaminated diet alone significantly (P≤0.05) decreased the testes weights at 6th weeks of age as compared to the controls. The supplementation of vinasse at 2.5%, 5% and 7.5% levels to the phenol contaminated diets improved the adverse effect of phenol on testes weight. This improvement in testes weight was significant (P≤0.05) in quail fed diet containing phenol plus vinasse at 7.5% level.

DNA Damage

DNA Fragmentation: The present results (Table 4 & Fig. 1) showed that the quail group fed diet contaminated with phenol had a significant (P≤ 0.05) increase in the rate of DNA fragmentation in their liver tissue as compared to the negative control group or the groups treated with vinasse alone. Addition of vinasse (2.5%, 5%, 7.5%) to the phenol contaminated diets decreased the rate of DNA fragmentation compared to the group fed the diet contaminated with phenol. However, these decreases were only significant by the supplementation of vinasse at a rate of 7.5% of the diet. The results also showed that the rates of DNA fragmentation were low and relatively similar in each of control group and quail groups fed basal diets treated only with the three levels of vinasse, with no significant differences for the frequencies of DNA fragmentation between these groups.

Gene Expression Analysis: The results of the IGFBPs gene expression in liver, testis and breast muscles tissues of quail are summarized in Figures 2, 3, 4, respectively. Compared to the negative control, the results revealed that the quail fed diet contaminated with phenol had significant decrease (down-regulation) of the expression level of IGFBPs gene in each of liver, testes and breast muscle tissues. In contrast, the supplementation of vinasse (5.0%, 7.5%) to the phenol contaminated diet ameliorated the expression level of the IGFBPs gene in liver, testis and breast muscles tissues as compared to the phenol contamination alone. These improvements of expression level of IGFBPs were significant by the addition of 7.5% vinasse to the diet. Also, statistical analysis showed that there were no significant differences between quail fed basal diet (without treatment) and quail fed basal diet treated only with vinasse (2.5%, 5% and 7.5%) for expression level of IGFBPs gene in all examined tissues. The only exception to this was a marked increase in the expression level of the IGFBP gene that was observed in quail fed the basal diet contaminated with 7.5% vinasse. However, this increase was only significant in the liver tissue.

Histopathological Results: The results of this study revealed that phenol has toxic effects on liver, testis and breast muscle tissues as compared to the control group. Histopathological examination of liver tissue sections from the control quail showed normal structure of this tissue composing of cords of hepatocytes radiating from a central vein (Fig. 5A). Liver sections from quail fed phenol contaminated diet (Fig. 5B) showed a marked dilatation and congestion of the main blood vessels with thickening of their walls. The phenol also caused vacuolar degeneration of hepatocytes. Histopathological examination of testis tissue section of the negative control quail showed seminiferous tubules with connective tissue in-between (Fig. 7A). Layers of germ cells lining the

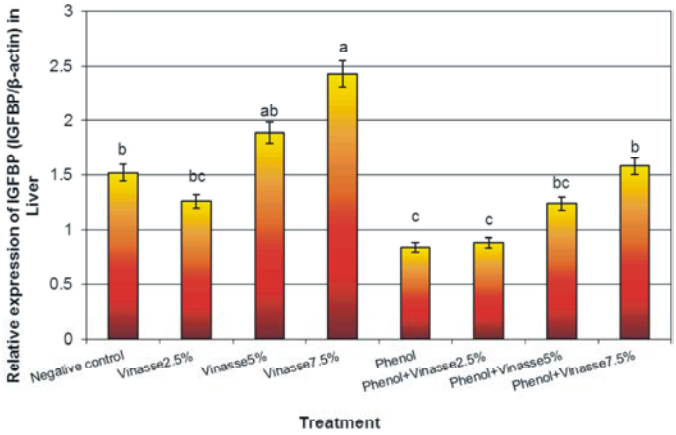

Fig. 2: Expression of IGFBP gene in the liver tissue of quail determined by semi-quantitative RT-PCR. The RNA recovery rate was estimated as the ratio between the intensity of IGFBP gene and the β-action gene
Means with different letters are significantly different (P≤ 0.05)

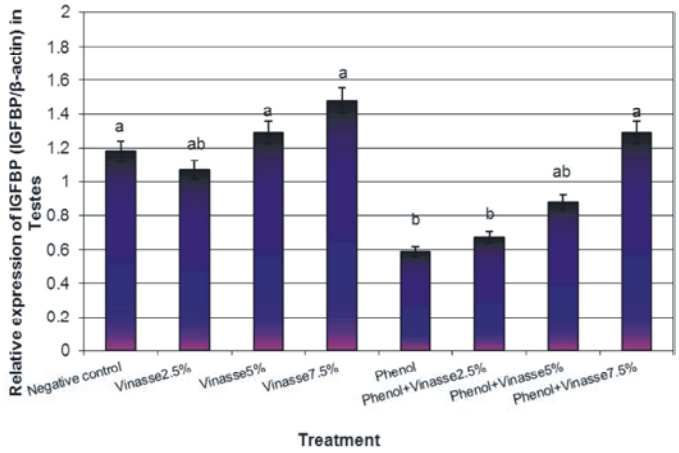

Fig. 3: Expression of IGFBP gene in the testis tissue of quail determined by semi-quantitative RT-PCR. The RNA recovery rate was estimated as the ratio between the intensity of IGFBP gene and the β-action gene
Means with different letters are significantly different (P≤ 0.05)

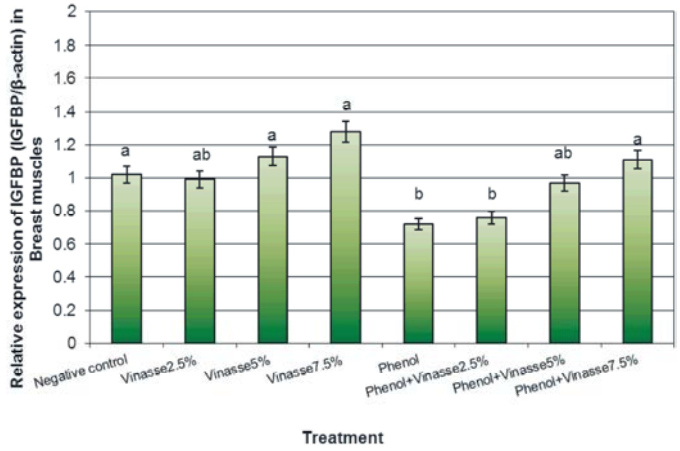

Fig. 4: Expression of IGFBP gene in the breast muscle tissue of quail determined by semi-quantitative RT-PCR. The RNA recovery rate was estimated as the ratio between the intensity of IGFBP gene and the β-action gene
Means with different letters are significantly different (P≤ 0.05)

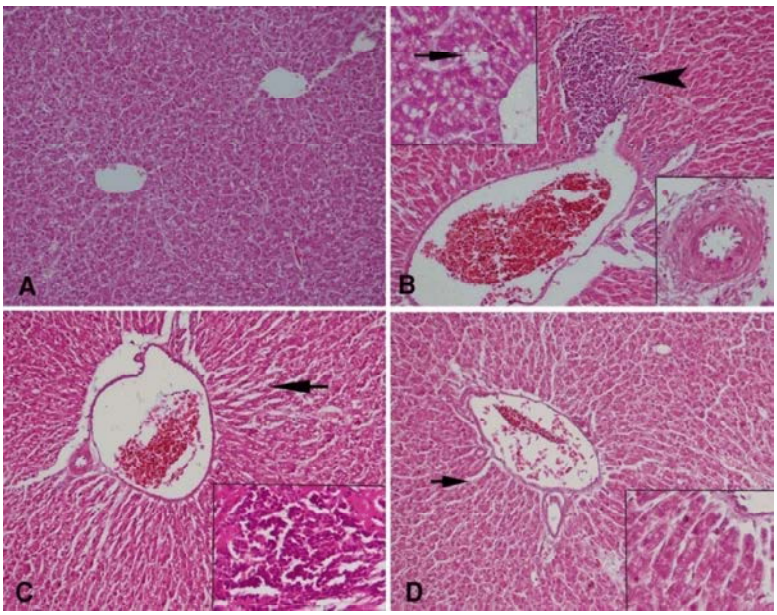

Fig. 5: Photomicrograph of sections of liver tissue (A) from a negative control quail shows the normal structure of this tissue composing of cords of hepatocytes radiating from a central vein. (B) phenol contaminated group shows severe dilatation with congestion of main blood vessels and focal aggregations of cellular infiltrates (arrowhead) at many places. The higher magnification parts show noticeable vacuolar degeneration of many hepatocytes (arrow) and marked thickening of blood vessels' walls. (C) liver tissue from a quail fed phenol and vinasse at a dose of 2.5% shows mild improvement, but with dilatation of blood sinusoids especially around portal areas. The higher magnification part shows focal aggregation of cellular infiltration. (D) liver tissue from a quail fed phenol and vinasse at a dose of 5% showing reduction of blood vessels' and blood sinusoids' dilatation (arrow). At a higher magnification no vacuolar degeneration of hepatocytes is observed. (Hx & E X100 & 200)

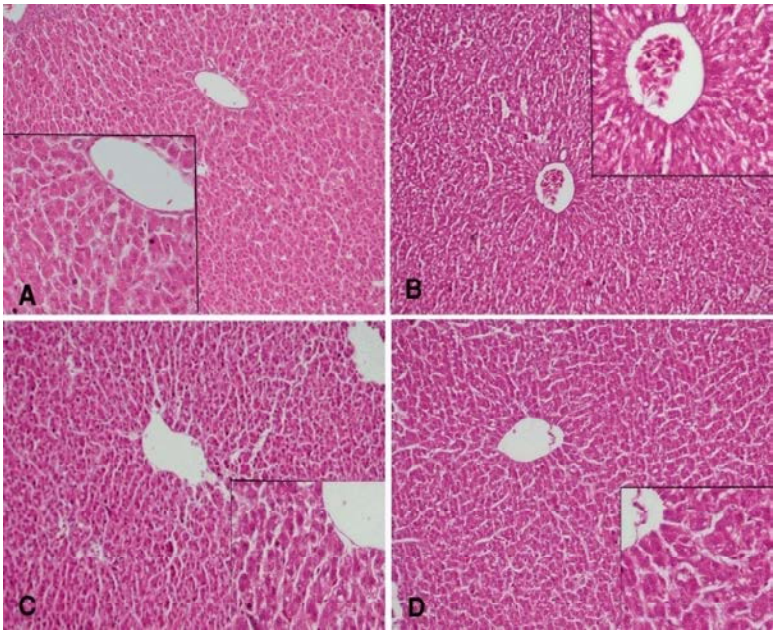

Fig. 6: A Photomicrograph of sections of liver tissue (A) from a quail fed phenol contaminated diet and vinasse at a dose of 7.5% showing normalization of liver tissue. (B) quail fed vinasse only at a dose of 2.5% showing normal liver tissue. The high magnification shows normal hepatocytes. (C) quail fed vinasse at a dose of 5% showing quite normal liver tissue. (D) quail fed vinasse at a dose of 7.5% showing the same results as the previous group

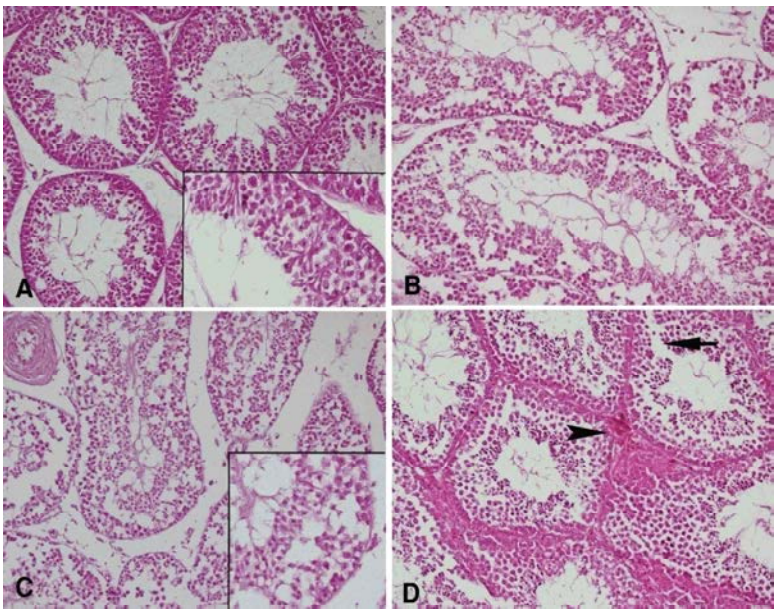

Fig. 7: A photomicrograph of sections of testicular tissue of quail (A) from a negative control testis showing the seminiferous tubules. The germ cells and sertoli cells with the sperms appear in the high magnification part. (B) phenol contaminated group shows severe damage in the form of large gaps between germ cells shedding the upper layers of cells. (C) Testicular tissue from a quail fed phenol contaminated diet plus vinasse at a dose of 2.5% showing deformation of the seminiferous tubules. The high magnification part shows vacuolar degeneration of the spermatogenic cells forming large gaps. (D) testicular tissue from a quail fed phenol contaminated diet plus vinasse at a dose of 5% shows reduction of the gaps between the spermatogenic cells (arrow) with thickening of the basement membrane of the tubules and congestion of interstitial blood vessels (arrowhead)

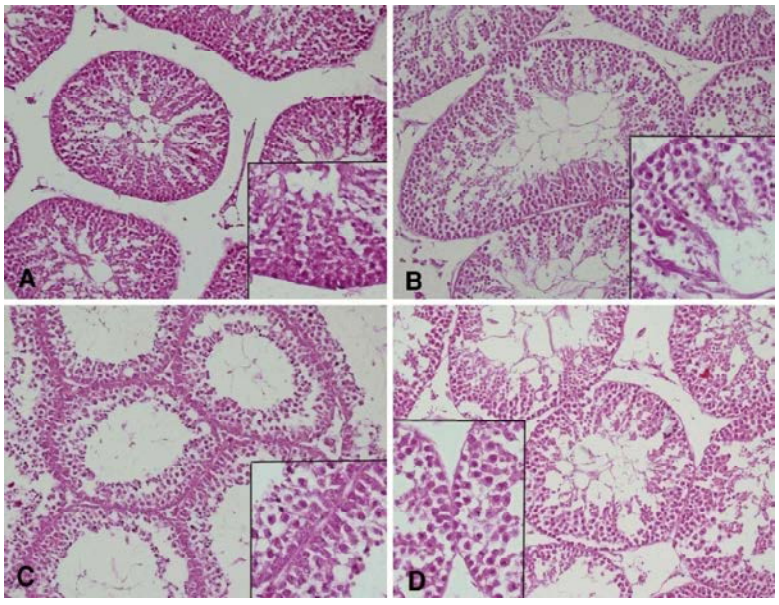

Fig. 8: A photomicrograph of sections of testicular tissue (A) from a quail fed phenol contaminated diet plus vinasse at a dose of 7.5% showing restoration of the normal structure of the tissue, but with increased connective tissue between the tubules. (B) testicular tissue from a quail fed basal diet plus vinasse at a dose of 2.5% showing normal appearance of tissue. (C) testicular tissue from a quail fed basal diet plus vinasse at a dose of 5% showing normal seminiferous tubules, but with no sperms attached to the sertoli cells.(D) testicular tissue from a quail fed basal diet plus vinasse at a dose of 7.5% showing the same results as the previous group

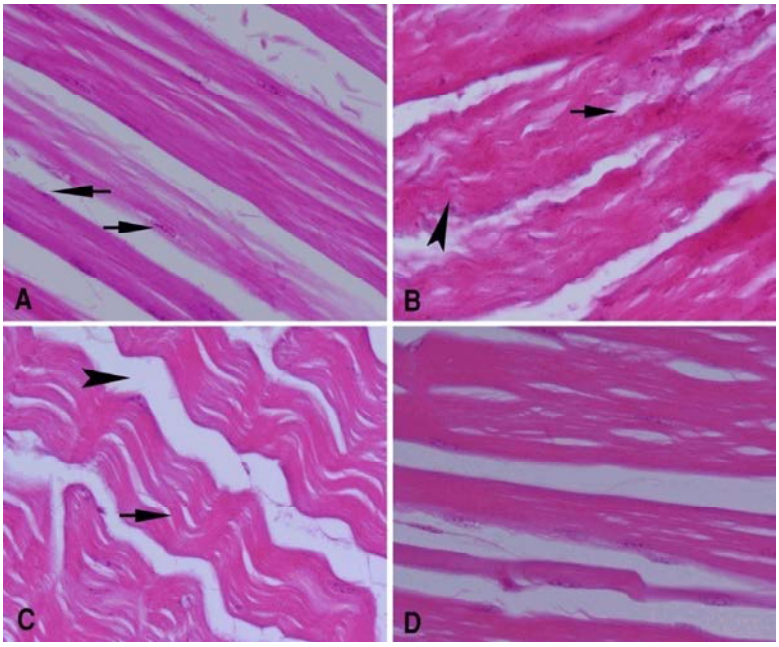

Fig. 9: A photomicrograph of sections of skeletal muscle fibers (A) from a negative control quail showing some fibers of normal width with peripheral flattened nuclei (arrow), just beneath the sarcolemma. (B) phenol contaminated group shows wavy muscle fibers (arrowhead) with gaps in between the fibrils (arrow). The nuclei show karyorrhexis. (C) quail fed phenol contaminated diet plus vinasse at a dose of 2.5% shows the wavy skeletal muscle fibers are still appeared (arrow). The nuclei are of normal size and shape. (D) quail fed phenol contaminated diet plus vinasse at a dose of 5% shows straightness of muscle fibers, some of them are of normal width, while others appeared atrophied

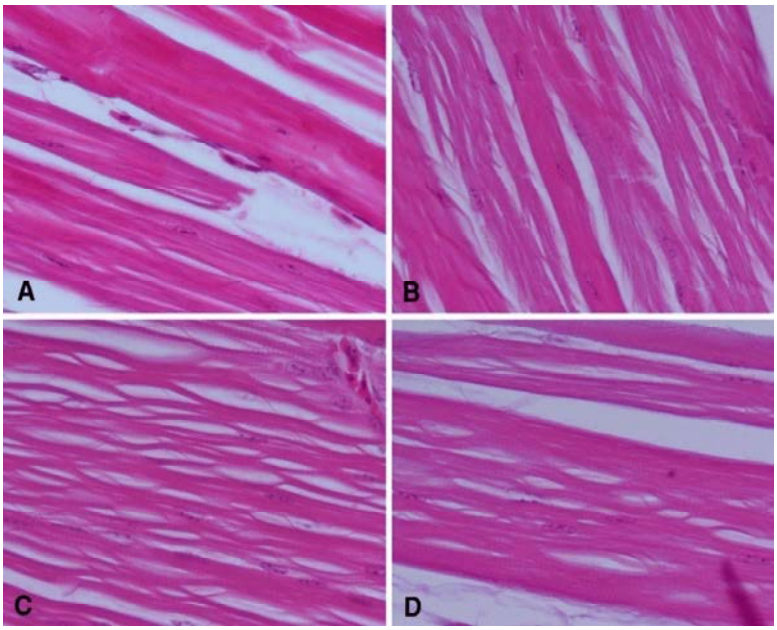

Fig. 10: A Photomicrograph of sections of skeletal muscle fibers (A) from a quail fed phenol contaminated diet plus vinasse at a dose of 7.5% shows skeletal muscle fibers close to normal. (B) quail fed basal diet plus vinasse at a dose of 2.5% shows normal skeletal muscle fibers, although some of them appear thinner than normal. (C) quail fed basal diet plus vinasse at a dose of 5% shows completely normal skeletal muscle fibers. (D) quail fed basal diet plus vinasse at a dose of 7.5% shows the same results as the previous group

tubules and sertoli cells with the sperms embedded in its apical part were also apparent. The testicular tissue from the phenol group fed contaminated diets (Fig. 7B) suffered from the damaging effects of phenol as large gaps appeared between the germ cells led to detachment of layers of spermatocytes and spermatids and disappearance of mature sperms. The histopathological examination of sections of breast muscle tissues (Fig. 8A) of a control bird showed some fibers of normal width with peripheral flattened nuclei; just beneath the sarcolemma. However, the phenol group fed contaminated diet showed damage to the skeletal muscle fibers in the form of waviness of the muscle fibers (Fig. 8B), the presence of gaps in-between the fibers and karyorrhexis of their nuclei. On the other hand, the histopathological examination showed that using vinasse as a protecting agent led to dose-dependent amelioration of the toxic effects of phenol on the examined organs, where the histopathological changes were restricted and improved in liver, testis and breast muscle tissues in comparison to those found in the phenol treatment alone. The supplementation of vinasse at level 7.5% was more effective in the protection than other two vinasse levels, (2.5% and 5%). These improvements of vinasse were described in Fig. 5 C, D and Fig. 6A on liver tissues, Fig. 7 C, D and Fig. 8 A on testis tissues and Fig. 9 C, D and Fig. 10A on breast muscle tissues. Moreover, the histopathological examination showed that the supplementation of vinasse to the basal diet (without phenol contaminated, as described in Fig. 6 B, C and D on liver tissues, Fig. 8 B, C and D on testis tissues and Fig. 9 B, C and D on breast muscle tissues), indicated no deleterious effects due to vinasse treatments. These results showed quite normal structure in the examined tissues.

DISCUSSION

Effect of Phenol Treatment on Productive Performance: In the present study, the quail consumed phenol (0.75gm/kg) containing diet showed poor body weight from 5th week to 6th week and poor body weight gain testes from 3 week until 6 week of age. The deleterious effects of phenol were statistically significant especially on BWG from 3 to 6 weeks of age. Moreover, feeding the quail phenol contaminated diet significantly decreased their liver and testis weights at 6th week of age as compared to the controls. The present findings indicated that phenol might induce generalized toxicity in the quail including its testes. To our knowledge, the role of phenol for inducing depression of BW and BWG as well as for decreasing organs weights in birds has not so far been studied. However, it was reported that the chronic exposure of workers to phenol vapours caused anorexia, weakness and lost of body weight [1, 41].

Also, it was stated that the phenol compounds are accumulated in liver, kidneys, brain, lungs and urogenital tract causing reduction in body weights [11-13]. The results of a clinical investigation [42] described mass poison with phenols. The example was pollution of water and fish in reservoir in Jarrela locality in South Finland with phenols derived from a wood processing plant. The adverse effects of phenol on BW, BWG and weights of liver and testes may be due to disruption in the activity of the digestive enzymes, inhibition of protein synthesis and the absorption of essential nutrients [1, 11- 13].

Effect of Phenol Treatment on DNA Damage: The present study showed that feeding birds with phenol contaminated diet led to significant increase ($P \leq 0.05$) in DNA fragmentation rate as compared to the negative control. Similar results were reported by Eshak *et al.* [43] who found that mice which fed diets contaminated with phenol compounds derived from sugarcane begass cellulose wood-mill, that resulted in significantly ($P \leq 0.05$) higher DNA damage (by using comet assay of DNA) as compared to the negative control. Other investigations showed that the exposure phenol or phenol compounds inhibits synthesis and replication of DNA in each of Hela cells [44] and diploid human fibroblasts [45].

Phenols were also found to suppress the enzyme activity of ribonucleotide reductase that participates in DNA synthesis [46]. The toxicity of phenols is considered to be the main factor for DNA damage, reactive oxygen species (ROS) and lipid peroxidation (LPO) during the metabolic processeces of phenols in liver. [1, 7 and 8]. Thus, phenols cause unscheduled DNA synthesis or DNA adducts formation and subsequently single and double strand scissor of DNA are produced [1]. Phenol compounds were found to be capable of interacting with genetic material causing damage to the DNA structure of mouse and human lymphocytes [1, 47].

Effect of Phenol Treatment on IGFBPs Gene Expression: The present results indicated that feeding birds with phenol contaminated diet caused significant ($P \leq 0.05$)

down regulation of the expression of IGFBPs gene in the liver, testis and breast muscle tissues as compared to the controls. Abnormal expressions of multidrug resistance and cytochrome P1A2 (CYP1A2) genes was found in kidney and liver tissues, respectively in mice fed diets contaminated with phenol compounds derived from sugarcane begass cellulose wood-mill as indicated by Eshak *et al*. [43]. Also, in a previous study [48], it was reported that the administration of phenol compounds in rats caused changes in the activity of the immunological system. The mechanism of action was related to modulation of genes expression that is responsible for mRNA synthesis in tymocythes. Decrease of mRNA synthesis inhibited the thymocyte proliferation. Many of the toxicants that lead to toxicity could also lead to DNA and protein damage as well as modified gene expression [49]. DNA damage can have biological consequence such as transcription and/or replication inhibition leading to abnormal gene expressions [1, 50, 51].

Effect of Phenol Treatment on Histopathological Changes: The present study indicated that phenol has toxic effects on liver. These toxic effects were in the form of marked dilatation and congestion of main blood vessels with thickening of their wall. These findings were supported by a report of Eshak *et al*. [43] who revealed that phenolic compounds caused dilatation of main blood vessels and blood sinusoids and vacuolar degeneration in most hepatocytes in mice liver.

Chronic administration of phenol by animals led to pathological changes in liver, lungs, kidneys, skin, esophagus and urogenital tract [1]. These changes are mainly induced by lipid peroxidation that is responsible for damage and finally degradation of cell's membrane. Moreover, our study showed that the testicular tissues were also damaged by the phenol in the diet as large gaps appeared between the germ cells leading to detachment of layers of spermatocytes and spermatids and disappearance of mature sperms. Our findings are in coincidence with those of Aoyama *et al*. [52] and Jung *et al.*[53], who stated that some phenols are capable of disturbing sexual hormones function, causing sterility cases of animals and humans. The examples are alkylphenols, bisphenol A, 2, 4- dichlorophenol and pentachlorophenol.

Also, in another experiment, it was reported that bisphenol A caused protein expressions in Tm4 cells in mice, which play a key role in spermatogenesis [54]. In this respect, the viability of cells decreased 10 to 70 % after exposure to doses of 50-250 µm/kg of body weight over 16 hours. These results showed that bisphenol A may induce infertility in mice.

There are some places within a receptor that may bind not only 17-β- hydroxyl groups of hormones, but also hydroxyl residues of phenols as well. Moreover, it is considered that core of alkylphenols imitates a ring A in E2 estrogens and thus reveal estrogenic activity. The present work also indicated that phenol caused damage to breast muscle fibers in the form of waviness of muscle fibers, the presence of gaps in between the fibrils and karyorrhexis of their nuclei. These results was explained by Hansch *et al*. [7] who reported that phenol exerts a marked corrosive action on any tissue of contact when ingested, inhaled or after skin exposure. Its cellular uptake is both rapid and passive due to its lipophilic character and signs of systemic toxicity develop soon after exposure.

Effect of Addition of Vinasse to Phenol Contaminated Diet on Productive Performance: The present results showed that the addition of vinasse to phenol contaminated diet improved the adverse effect of phenol on BW, BWG and weights of liver and testes. These findings indicate the effect of vinasse on detoxification of phenol. To our knowledge, the role of vinasse in detoxification of any toxicants has not been studied yet. However, these positive effects of vinasse might be due to its contents of yeasts, minerals, vitamins and organic acids [19, 29]. These compounds were found to be strong antioxidants [22, 23 , 27 and 28] which have the ability to produce biological enzymes that interact with toxicants [55, 56].

It was also reported that yeast has been known to alter stress in animals by providing a source of vitamins, enzymes and growth protein for reducing stress and to enhance the biological value of nitrogen compounds along the digestive tract [55]. Moreover, the polysaccharides, beta-glucans and manan type in the cell wall of yeast can exclude pathogens at the digestive tract of the chicken [19, 30]. In response to these effects, the development of the digestive mucosa is favored and a better state of immunocompetency is maintained in the bird, leading to improvement in the productive and reproductive performance [19]. In another study by Abousadi *et al.* [57], it was observed that the addition of yeast of *Saccharomyces cerevisiae* (Sc) to

AFB_1- containing diet significantly improved the adverse effect of AFB_1 on growth performances in broiler chicks. Sc supplementation to quail diets suppressed the aflatoxicosis in quail tissues leading to improvement of BW and BWG as concluded by Eshak *et al*. [43].

Effect of Addition of Vinasse to Phenol Contaminated Diet on Genetic Alterations: The present results showed that the vinasse was able to improve the genetic alterations induced by phenol in the quail tissues. Where, the rate of DNA fragmentation in liver tissues was decreased and the expression level of IGFBPs gene was ameliorated in liver, testes and breast muscles tissues in quail fed diets contaminated with phenol plus vinasse as compared to treatment with phenol alone. These improvements were more pronounced at 7.5% vinasse/diet than any of the other vinasse levels. To our knowledge, the use of vinasse as a protective agent against the mutagenic effects of toxicants has not been discussed previously. However, the antimutagenic activity of vinasse which was observed in the present study might be due to its contents of high amount of antioxidant constituents as reported above by Hidalgo *et al*. [29] and Mc-Pherson *et al*. [19]. These constituents are considered to be as an antimutagenic agents, that interrupts the free radical-initial chain reaction of oxidation or scavenge and disable free radicals (ROS) and reduced DNA oxidative damage [26, 28, 58, 59] leading to genomic stability which include expression of animal genes [60-62].

Also, Five peroxiredoxins named Tsal (CTPX1), Tas2, AhP1, Dot5 and Prx1, of which Tsal possesses the most potent ability to scavenge H_2O_2 was identified by Park *et al*. [63] in the yeast (that is a main constituent of vinasse). Moreover, it was reported by Huang *et al.* [64] that the Tsal is the most potent protector of genomic stability and prevents a broad spectrum of mutations. Furthermore, the expression levels of neural and gonadal genes were significantly up-regulated in quail fed diet containing AFB_1 plus yeast of *Saccharomyces cerevisiae* as compared to those of AFB_1 group as reported by Eshak *et al*. [65].

Effect of Addition of Vinasse to Phenol Contaminated Diets on Histopathological Changes: The present findings showed that the addition of vinasse to phenol contaminated diets restricted and improved the histopathological changes leading to better results in comparison to those found in the phenol contamination alone. The supplementation of vinasse at 7.5% level was more effective in the protection than the other two vinasse levels (2.5% and 5%). To our knowledge, the protective role of vinasse for histological architecture against deleterious effects of toxicants has not been discussed previously. However, *Saccharomyces cerevisiae* (Sc) which is a main constituent of vinasse, could exert effects on the immune system of the chicken and exclude of pathogens of digestive scale [30]. Moreover, it was concluded by Darwish *et al*. [66] that the supplementation of Sc yeast to mice diets suppressed the aflatoxicosis and the generation of (ROS) in animal tissues by ameliorating the GSH level and SOD activity. The improvement of such agents led to enhancement in liver and kidney tissues architecture.

In this respect, it was reported by Ozcelik *et al*. [67] that the decrease in SOD activity will increase ROS activity and lipid peroxidation (LPO) level. The ROS and LPO agents have been found to play an important role for causing liver and kidney damages [66], carcinogenesis, aging and a variety of mammalian diseases [27]. For example, cancer susceptibility is frequently a pathological consequence of intensive and sustained ROS-related damage in chronic infection and associated inflammation [68, 69]. Perturbation of the cellular balance of ROS clearly contributes to specific aspects of clinical and cellular phenotypes, in particular to degenerative changes in specific tissues and premature aging.

Effect of Addition of Vinasse to the Phenol-Free Basal Diet: The addition of vinasse (especially at the level of 7.5) to the basal diet without phenol contamination did not alter BW, BWG, weights of liver and testes and expression level of IGFBPs gene in liver, testes tissues and breast muscles. Some improvements were significant from the negative controls in BW, BWG and expression level of IGFBPs gene in liver tissues. However, there were no significant differences between quail fed the basal diet (control) and quail fed basal diet supplemented with different levels of vinasse for the frequencies of DNA fragmentation. Also, histopathological results were relatively similar in the control and vinasse groups. These results showed quite normal structures in the liver, testis and breast muscle tissues.

The effect of vinasse as a feed additive was discussed previously only on poultry performance. It was proved by Sarria *et al*. [70] that there was improvement in the growth rate in birds of about 5% and a decrease the

price of the ration by 15%, through the addition of concentrated vinasse between 2% and 3% in the diets. Also, improvement in the weight of the reproductive tract and the follicle count in pullets that used the distillery vinasse, as food additive, was observed by Nakano *et al.* [71]. In pullet rearing, the inclusion of vinasse in the diet had produced alterations in the intestinal microflora, increased the digestibility and absorption of nutrients and led to a greater viability, with better results at 2% inclusions [72].

It was reported by Hidalgo *et al*. [19] and Javierre [73] that live-weight, weight gain and mortality were better in birds that consumed combinations of acidifying supplements of vinasse (2%) in the diets. These positive results of vinasse could be explained by Mc-Pherson *et al.* [29] who reported that the importance of vinasse on productive performance might be due to its contents of organic acids, yeast walls and vitamins especially B-complex vitamins. These compounds were found to increase the efficiency of utilization of nutrients and therefore produce a better performance of birds or animals. Improvements in the performance of the thighs and abdominal fat reduction, as well as a trend towards breast weight improvement in chickens that consumed the brewing yeast supplement were observed by Miazzo *et al*. [74] and Miazzo *et al.* [75].

In conclusion, our results proved the protective effects of vinasse against the harmful effects of phenol contaminated quail feed. These positive effects of vinasse (7.5%) supplementation in quail rations, especially in case of phenol contamination suspicion, suppressed the toxic effects of phenol, reduced DNA damage, improved IGFBPs gene expression, ameliorated histopathological changes of several organs and consequently enhanced the productive performances of the birds.

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