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Original Article

Alteration of PUMA gene expression in mice by royal jelly and green tea

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ABSTRACT

Aim: The purpose of this study examine whether the polymorphism in (TNF-α) gene correlated with activate p53 upregulated modulator of apoptosis (PUMA) gene consequential exposure to pesticides and the effects of green tea and royal jelly in rats. **Methods:** Experimental groups were organized into 12 groups including 10 animals per each. Contain control group, pesticide group and the rest of groups were pesticide + protective. Blood samples were collected form rats before and after occupational exposure to pesticides for liver and kidney enzymes biochemical analysis, the polymorphism of the genes for TNF-α and PUMA were typed from genomic DNA and Allelic frequencies and carriage rates were compared between healthy and treated rats. The correlation between TNF-α and PUMA genotypes and the clinical characteristics of rats were also evaluated. **Results:** Data statistical analysis for mitochondrial enzymes in our study showed a very highly significant (P>0.001) difference in the mean value of serum enzymes isolated from treatments groups with pesticides before and after Royal jelly and Green tea treatments. The results of restriction enzymes for TNF-α showed there are no different between negative control with and both of two protective agents in the results of restricted bands. But the results from PUMA showed a strong deferent between the treatments with royal jelly and green tea combined with pesticides. The royal jelly and green tea enhance the repair system enzymes to remedy the mutation that originated in DNA after exposure to pesticides. **Conclusion:** Our recent work provides direct evidence Royal jelly and Green tea could be affects on mechanism related to the repair system in cell to remedy the DNA mutation

KEY WORDS: PUMA, Gene, Expression, Royal jelly, Green tea

INTRODUCTION

The pesticides are one of the most potentially harmful chemicals liberated in the environment in an unplanned manner[1]. Dimethoate is widely used as a potent pesticide in many countries and has been shown to produce some adverse health effects.A great proportion of acute poisoning cases are caused by exposure to pesticides, especially organophosphate (OP) compounds. The primary mechanism of action of OP pesticides is based on inhibition of the acetylcholinesterase (ache) enzyme [2].

Chlorpyrifos is a colorless to white crystalline solid. Chlorpyrifos has a mild mercaptan (thiol) odor, similar to the smell of sulfur compounds found in rotten eggs, onions, garlic and skunks. [3]. It can be absorbed easily through the gastrointestinal mucosa, lung epithelium, and skin and Alzheimer di s-ease [4].

Methomyl is a broad spectrum carbamate insecticide its high solubility in water and low affinity for sediment binding, it may have potential for ground-water and surface water contamination [5]. Methomyl is highly soluble in water and a low sorption affinity to soils and can herefore easily cause groundwater contamination in agricultural areas[6].

Royal jelly has received particular attention because of studies that have reported that it is a highly efficient antioxidant and has free radical scavenging capacity [4, 7]. Royal jelly is a secretion produced by the hypo pharyngeal and mandibular glands of worker honeybees (Apis mellifera). It contains many important compounds with biological activity such as free amino acids, proteins, sugars, fatty acids, minerals, and vitamins [3, 8].

Green tea (GT) is one of the most ancient beverages, consumed by over two-thirds of the world's population. The principal constituents are caffeine, tannins, and essential oils [9]. GT compounds are chemically classified as dibenzpyrans, pyrones, and their derivatives. The core structure contains a diphenylpropane skeleton. The primary flavonoids found in fresh green tea leaves are catechins (flavan-3-ols or flavanols) and the flavonols [10]. The most abundant polyphenolic compound is EGCG, thought to contribute to the beneficial effects attributed to green tea, such as its anticancer, cardiovascular function improvement and antioxidant anti-inflammatory properties [11].

Tumor necrosis factor-alpha (TNF-α) was discovered 30 years ago as a product of immune activation[12]. In the last three decades, an impressive amount of knowledge has been obtained regarding the biological functions of TNF, as well as the signaling mechanisms engaged by its receptors. TNF primarily occurs as a type II trans membrane protein of 26 kDa, which can be cleaved by the metalloprotease TNF-a-converting enzyme to a 17 kDa. Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine involved

in the promotion and progression of cancer [13]. It plays an important role in the tumor microenvironment both as a membrane-integrated protein and in its soluble form generated after proteolytic cleavage [14].

PUMA is a BH3-only Bcl-2 family protein that mediates both p53-dependent and independent apoptosis. However, its role in tumor suppression had not been well established [15]. It is normally expressed at a low level but is markedly induced after cells are exposed to DNA-damaging agents, such as chemotherapeutic drugs and ionizing radiation [16], and play an essential role in p53-dependent and -independent apoptosis induced by a variety of stimuli [17]. Programmed cell death, or apoptosis, is important for the development and homeostasis of tissues. Too little cell death can result in autoimmune diseases or cancer, whereas excessive cell death can lead to debilitating degenerative diseases of the heart or nervous system. The realization that apoptosis was genetically controlled first arose when it was observed that certain mutants of the model organism Caenorhabditis elegans caused failure of apoptosis in cells that normally undergo this process during development [18].

MATERIALS AND METHODS

Chemicals

Fresh green tea powder was purchased locally. The crude aqueous extract of green tea was prepared by soaking 30 g of instant green tea powder in 1 L of distilled water whose temperature did not exceed 90oC, for 5 min to obtain soluble polyphenols dissolved in the aqueous extract. The solution was filtered to obtain the final 3% green tea extract. This solution was orally injected to rats daily for 28 day and another concentration was made 4.5% and 6% by the same way. Royal jelly was purchased from (Agriculture Research Center). Royal jelly dissolved in distilled water. Three concentrations of royal jelly were made [19]. Methomyl pesticide and chlorpyrifos pesticide were obtained from Agricultural research Centre- central laboratory for pesticide. Methomyl dose was prepared at concentration of 1/10 of LD50, and LD50 of methomyl is 20 mg /kg .then take 2 mg /kg for each rate as daily dose dissolved in 1 ml of distilled water. Methomyl were taken for 21 day. Chlorpyrifos dissolved in corn oil. Chlorpyrifos dose was prepared at concentration of 1/10 of LD50, and LD50 of Chlorpyrifos is 138 mg /kg so we take 13.8 mg /kg for each rate as daily dose dissolved in 1 ml of corn oil. Chlorpyrifos were taken for 21 day.

Experimental animals

The experimental animals used in this work were 72 random bred adult males of laboratory albino rats (140-150 g in weight). They were maintained under standard condition and fed standard chow and water adlibitum. All experiments were carried out in accordance with protocols approved by the local experimental animal ethics committee. Administrated intraperitoneal. Rats were sacrificed 24 h post-injection and grouped.

Experimental design

A three dose (100,200,300 mg/kg) of Royal jelly and (3, 4,6mg/Kg) of Green tea were selected as a protective. Experimental groups were organized into 12 groups including 10 animals per each. Contain control group, pesticide group and the rest of groups were pesticide + protective.

Biochemical analysis and oxidative stress

different parameters (SGOT, SGPT, Albumin, Creatinine, Cholestrole, LDH, Urea, Alkaline phosphatase) were measured for each rat in each group . for oxidative stress three parameters were measured (GSH for liver and kidney, SOD for liver and kidney, MDA for liver and kidney) for each rat in each group .

PCR Amplification

DNA was extracted from kidney tissue and analyzed .PCR primers pair was designed by (Biolegio BV) for the following genes .TNF gene F: ggtctgatttgcagggactt and R: ccagtgaacggacggctaaT. For PUMA geneF: caactaggtgcctacacccg and R: aggctagtggtcaggtt, each gene was amplified with specific protocol.

Digestion with Restriction enzymes.

The genes were digested with restriction enzyme to describe polymorphism between control, pesticide and protective groups. TNF gene with SacII (cfr421 fast digist). PUMA gene was digested with HpaII. BECLIN gene was digested with SacII (cfr421 fast digist), HpaII, SmaI.

Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Tables 1 and 2 depict the effect produced on selected functional indices of rat liver and kidney respectively following the repeated administration of yohimbine. Yohimbine administration resulted in an initial significant decrease in serum content of bilirubin ($P<0.01$) and by the fifth day, the concentration has increased significantly when compared with the control ($P<0.01$) (Table 1). The bilirubin concentration compared favourably with the control by the tenth day of administration, a trend which was not sustained beyond that day as there was significant reduction in bilirubin concentration of about half the control value ($P<0.01$) (Table I). The serum albumin concentration displayed a general pattern of significant increase right from the start of the drug administration and was sustained throughout the experimental period ($P<0.01$).

RESULTS

In the present study, acute kidney toxicity was induced in male Albino rats' by using Methomyl and chloropyrifos pesticide. The protective effect of green tea and royal jelly

was tested by orally administration for 7 constitutive day's before pesticide. Biochemical and molecular parameters were used to assess the protective effect of green tea and royal jelly.

Demographic and clinical information of patients and controls

The general demographic characteristics of the subjects and the most commonly enzymes used indicators of liver (hepatocellular) damage are the ALT, alanine aminotransferase, AST, aspartate aminotransferase, ALB, albumin ;ALP, alkaline phosphatase and for kidney function CREAT, creatinine; CHOLE, cholesterol; LDH, lactate, data represented the statistical analysis and mean values of different enzymes level in the serum of two groups G1, group I(methomyl + royal jelly); G2, group II (chlorpyrifos + green tea) of the present study . Highly significant (P>0.001) reduction in the mean value of serum AST was observed in the serum of rats treated with methomyl when compared either to control, data represented in table (1) and figure (1). On the other hand, AST level exhibits a significant increase (P<0.001) in protected group of animals with royal jelly when compared to that of methomyl group, the same results found in ALP, alkaline phosphatase, LDH, lactate dehydrogenase and Urea, conversely the control less than the group values with ALT and cholesterol and and no significance with Creatinin and ALB.

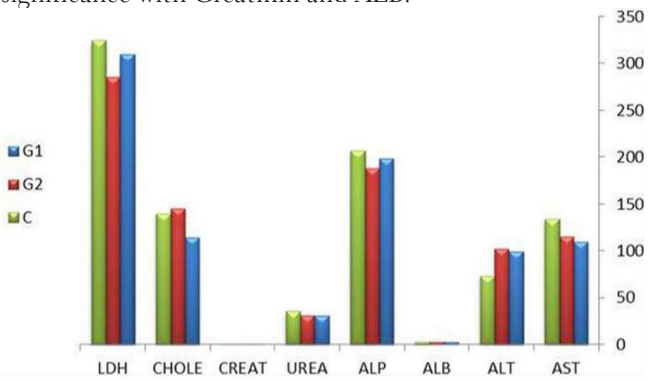

Figure 1. ALT, alanine aminotransferase; **AST,** aspartate aminotransferase **; ALB** ,albumin **; ALP** alkaline phosphatase **; CREAT,** creatinine ; **CHOLE,** cholesterol **; LDH,** lactate dehydrogenase ; Gl, group I(methomyl + royal jelly); G2, group II (chlorpyrifos + green tea); C, control.
****: Statistically significant at p i 0.001; F, factor**

Oxidative stress results:-

Group I: pesticide (Methomyl) / protective (royal jelly)

Superoxide Dismutase (SOD) activity in liver & kidney homogenate:

As shown in figure (2), the activity of superoxide dismutase (SOD) was significantly increased (P>0.001) in the liver and kidney homogenate of chloropyrifos rats as compared with normal control animals group. On the other hand, SOD activity was decreased significantly (P>0.001) in liver and kidney homogenate of normal control and green tea groups of animals as compared to that of chloropyrifos group of animals. Moreover, normal rats that maintained on orally administration of green tea for 8 constitutive days exhibited a significant increase (P>0.001) in SOD activity when compared to that of normal control group.

Reduced Glutathione (GSH) content in liver & kidney homogenate:

As shown figure (3), the GSH content was significantly increased (P>0.001) in the liver &kidney homogenate of methomyl rats as compared with normal control animals group. On the other hand, GSH content was increased significantly (P>0.001) in liver &kidney homogenate of green tea group of animals as compared to that of chloropyrifos group of animals. Moreover, normal rats that maintained on orally administration of green tea for 8 constitutive days exhibited a significant increase (P>0.001) in GSH content when compared to that of normal control group.

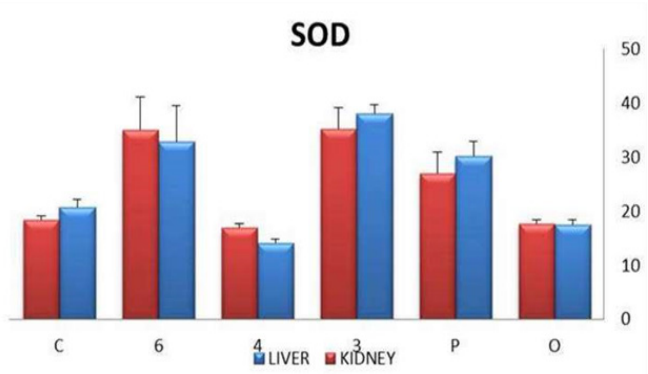

Figure 2. Level of SOD activity in the liver and kidney homogenate of different animal groups.

Table 1. Demographie and information of biochemical assays

	ALT		AST		ALB		ALP		CREAT		UREA		CHOLE		LDH	
	Gp1	Gp2	Gp1	Gp 2	Gp1	Gp 2	Gp1	Gp 2	Gp1	Gp 2	Gp1	Gp 2	Gp1	Gp 2	Gp1	Gp 2
	99.822	101.97	109.67	114.88	2.98	2.81	198.66	188.11	0.814	0.69	30.66	31.14	114.77	145.22	309.54	285.1
C	73.33		133.72		3.17		206.71		0.685		35.40		139.68		324.68	
F	5.42	25.55	6.210	10.881	13.36	3.661	4.796	4.812	5.41 1	5.520	8.456	3.751	7.491	7.894	9.222	2.985
P	.001	.000	.000	.000	.000	.010	.002	.002	.001	.000	.000	.009	.000	.000	.000	.050

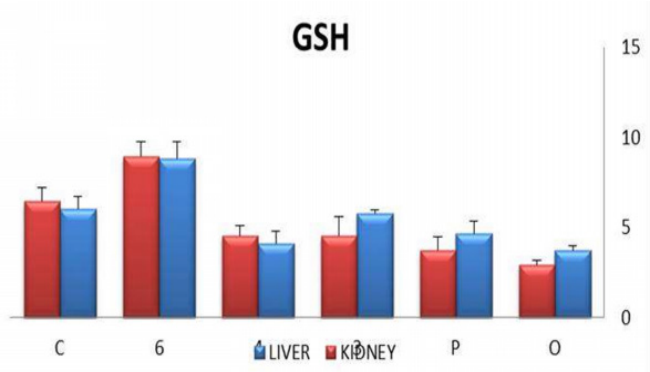

Figure 3. GSH content in the liver &kidney homogenate of different animal groups.

Level of lipid peroxidation in liver homogenate:

Figure (4) illustrate the level of malodialdehyde (MDA) as the end product of lipid peroxidation in liver & kidney homogenate. Results showed a significant decrease in lipid peroxidation level (P>0.01) in the liver homogenate of chloropyrifos rats as compared with normal control animals group. On the other hand, MDA was decreased significantly (P>0.01) in liver &kidney homogenate of green tea group of animals as compared to that of chloropyrifos group of animals. Moreover, normal rats that maintained on orally administration of green tea for 8 constitutive days exhibited a significant decrease (P>0.01) in lipid peroxidation level when compared to that of normal control group.

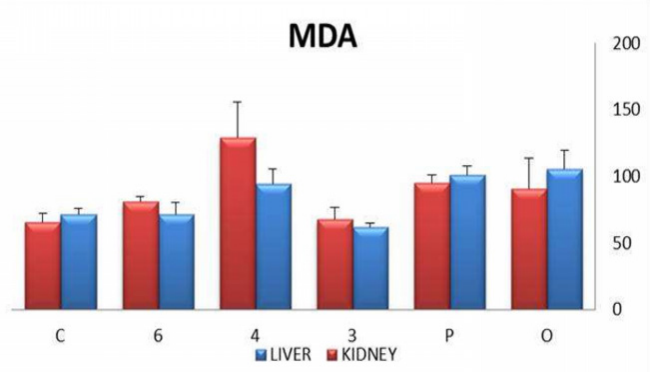

Figure 4. MDA concentration as the end product of lipid peroxidation in the liver and kidney homogenate of different animal groups.

Identification of PUMA and TNF-α

DNA extracted from kidney of different animal groups and after agarose gel electrophoresis of DNA extracted. Results showed all groups have a good extracted DNA there is no difference observed between protective group, normal control group and pesticide groups. PUMA and TNF- progresses through identifiable phases, which are differentially sensitive to treatments intervention. Therefore; we treated mice with the Methomyl, Chloropyrifos according to standard protocol and used the Green tea and Royal Jelly as a protective against the pesticide effects. To explore the functional relevance of the polymorphisms in PUMA and TNF- functional gene, we used the Restriction enzymes method to determine PUMA and TNF- expression different in genomic DNA treated mice for the genotype-phenotype correlation analysis between different protective treated of the two polymorphisms. In this analysis, the genotype was associated with the kind of treatment, the protective agent and the expression level with HpaII digested (restriction enzyme that cuts DNA at this recognition site: C^CGG), Fig. 5). To determine the potentially differential regulation of the PUMA activity, we further performed the digestion to verify whether these polymorphisms may change the activity of PUMA and TNF. We found there is no changing in the TNF- gene on the different levels of treatments and PUMA gene had a stronger change with the HpaII digestion, indicating a potentially different activity affinity of PUMA protein structure polymorphism (Fig. 6). These results might only suggest a potentially functional correlation between the protective agents (Royal jelly and Green tea) and PUMA expression and might not indicate the exact changes of PUMA nucleotide sequences. Therefore, further functional studies for the nucleotide sequences are needed to elucidate how and what extent the polymorphisms in PUMA may affect PUMA expression.

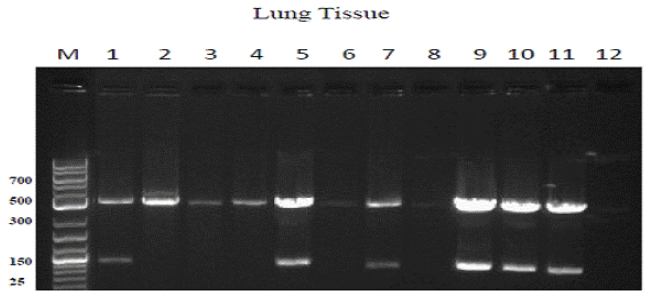

Figure 5. Agarose gel electrophoresis of digestion of PUMA gene with Hpall:-
(1) Negative control, (2) Methomyl treated Lung tissue, (3) C hlorpyrifos treated Lung tissue, (4) Methomyl treated lung tissue , (5) Methomyl treated lung tissue + Green Tea, (6) Chlorpyrifos treated Lung , (7) Chlorpyrifos treated Lung + Green Tea , (8) Chlorpyrifos treated Lung , (9) Chlorpyrifos treated Lung + Ro yal jelly, (11) Methomyl treated lung tissue , (12) Methomyl treated lung tissue + Royal j elly.

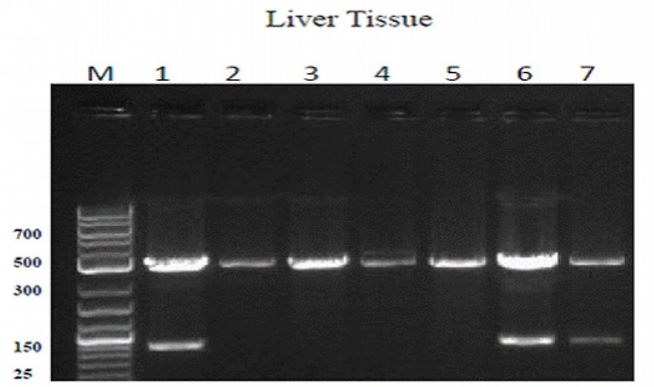

Figure 6. Agarose gel electrophoresis of digestion of PUMA gene with Hpall:-
(1) Negative control. (2.3) Methomyl tx eated Liver tissue. (4.5) Chlorpyrifos tr eated Lung tissue, (6) Methomyl treated liver tissue + Green Tea. (7) Chlorpyrifos treated liver+ Royal jelly.

DISCUSSION

PUMA is activated by transcription factors in response to stress, leading to apoptosis induction and tumor suppression. DNA damage or activated oncogenes induces PUMA through p53 to promote apoptosis. Inflammatory cytokines induces PUMA through TNF-α to promote apoptosis. DNA damage [2, 5], inflammatory cytokines and perhaps yet-to-be identified mediators and transcription factors (TF) might provoke PUMA-dependent apoptosis during inflammation, here we study the correlation between the polymorphism in TNF, PUMA genes and the protective role of Royal jelly and Green tea in mice treated with pesticides and relation with physiological enzymes related to mitochondria. Alanine transaminase, (ALT) and aspartate transaminase are enzymes that help in metabolism of protein [17, 20]. When the liver is damaged, ALT is increased in liver and released in the bloodstream. An increase in AST levels may indicate liver damage or disease. Aspartate transaminase is the mitochondrial enzyme, predominantly found in the liver, skeletal muscles and kidneys. Alanine transaminase is a cytosolic enzyme, which is more specific for the liver than aspartate transaminase [20]. In our study there was a highly significant difference in the parameters of biochemical analysis among 2 groups > 0.001 in all parameters except LDH >0.05, and ahighly statistical significance in parameters of oxidative stress (SOD, MDA, GSH) among all groups except MDA>0.05. The toxic effect of organophosphorus insecticides is to conjugate with the natural complement of enzyme in the body, thereby inactivating them. Organophosphorus pesticides (Monocroptophos, methyl parathion), caused significant in liver enzyme (aminotransferases, acid phosphatases. and alkaline phosphatases). However, phosphate enzymes act by hydrolyzing phosphomonoester including 3, and 5, phosphoproteins and these may also be involved in the transfer of phosphate, phosphatases are involved in many different processes that require mobilization of phosphate ion or dephosphorylation as part of anabolic, catabolic or transfer processes [4, 5, 21]. Transaminases are the metabolic enzymes which collect the amino groups from many different amino acids in the form of only one C-glutamate. The glutamate channels amino groups either into biosynthetic pathways or into a final sequence of reactions by which nitrogenous waste products are formed and then excreted [22, 23]. In the present study, the increased level of phosphatases and aminotransferases in blood may be indicate to metabolic activity, perhaps to meet the stress induced by prolonged exposure to the pesticides . The non-definite pattern of serum bilirubin concentration observed in the first five days of administration, may be attributed to various attempts by the tissue to adapt to the effect of the treatments, which was eventually achieved by the tenth day of administration. Consequently, the hypobilirubinemia is an indication of impairment of the tissue's functional capacity as extensive liver damage may lead to decrease serum levels of bilirubin [9, 24]. Liver is the exclusive site of synthesis of albumin. It may also be adduced to increased rate of hepatic synthesis of albumin without a proportionate increase in the rate of its catabolism. Consequently, the amino acid pool will no longer be maintained within normal limits. The hyperalbuminemia may adversely affect the transportation of a wide variety of ligands to the organs and tissues for their utilization or excretion. The observed hyperalbimnemia throughout the royal jelly and green tea may be an indication that the increase in albumin synthesis is dependent only upon initial administration and not the duration. The functional capacity of the kidney can be measured by the dye excretion tests, clearance test, concentration and dilution tests and method for examination of blood concentrations of excretory and electrolyte constituents [25, 26]. Furthermore, renal function tests are required either to demonstrate the presence or absence of active lesion in the kidney, or to assess the normal functioning capacity of different parts of the functioning unit, nephron. Inorganic electrolytes occur in large quantities in both extracellular and intracellular fluids. Due to their ability to dissociate readily into their constituent ions or radicals, they comprise the single most important factor in the transfer and movement of water and electrolytes between three divisions of the extracellular and intracellular compartment [23, 27]. However, the no significant difference from the control value by the end of royal jelly and green tea administration may be that the system has successfully combated the adverse effect of pesticides. Urea is the major nitrogen-containing metabolic product of protein catabolism. The significant reduction in serum urea concentration throughout the experimental period may be attributed to impairment of the urea cycle leading to reduced production of the metabolic product [28, 29]. The significant reduction in creatinine, another product of protein metabolism from the start of administration to half the control value by the end of administration may be an indication of compromise of the renal function. Royal jelly might have adversely interfered with the metabolism of creatinine leading to its observed reduction, an indication of partial loss of its functional capacity of tubular excretion [21]. The various alterations in the functional indices of the tissues and their values which do not compare favorably with their control values are clear manifestation of adverse effects of the functional parameters evaluated following the daily administration of the aphrodisiac. This study suggests that Royal jelly and green tea administration has good effect on the basic functions of the liver and kidney investigated [25].

These enzyme changes are indicative of the cellular toxicity and tissue damage induced by these pesticides in the rat probably by altering the specific molecular pathways. This is concomitant with results obtained. In this study, we found that there is polymorphism in digestion of TNF and PUMA genes. The results of restriction enzymes for TNF-α showed there are no different between negative control with and both of two protective agents in the results of restricted bands. But the results from PUMA showed a strong deferent between the treatments with royal jelly and green tea combined with pesticides, so the first explanation was the epigenetic modifications include DNA methylation, histone modifications, and microRNAs [8, 20]. DNA methylation is a covalent modification, involved

in reg-ulating many cellular processes including chromatin structure and remodeling, X-chromosome inactivation, genomic imprinting, chromosome stability, and gene transcription [30, 31] Post-translational modifications of histone tails have been shown to be important in altering chromatin structure and therefore DNA accessibility [23]. The functional effects of such modifications depend on the specific amino acid that is modified and on the specific covalently attached group: e.g. acetylation results in the loosening of chromatin and lends itself to replication and transcription, whereas methylated histones tight DNA and restrict access to various enzymes. Histones modifications can regulate gene expression, chromatin remodeling, cell survival and cell death [32, 33]. microRNAs (miRNA) are single-stranded RNAs of about 21–23 nucleotides in length that are transcribed from DNA but not trans-lated into proteins (non-coding RNAs). Their functional role is gene expression regulation mediated by a control of messenger RNA (mRNA) stability or translation. Exposure to pesticides may lead to epigenome modifications. Experimental, clinical, and epidemiological studies of epigenetic changes caused by pesticides exposure have increased our understanding of the mechanisms of action by which they can modify gene expression [34]. But could be proof this difference related to the epigenetics because we did not apply the bisulfide treatment for the isolated genomic DNA. One possible reason for the differences in the magnitude of changes could be that, as mentioned above, our results did not attributable to the epigenetics, the possible explanation the repair system. The royal jelly and green tea enhance the repair system enzymes to remedy the mutation that originated in DNA after exposure to pesticides. Lack of PUMA induction leads to chemo or radio resistance, while elevated PUMA expression induces pro-found chemo or radio-sensitization of cancer cells [35, 36]. PUMA PUMA is activated by transcription factors in response to stress, leading to apoptosis induction and tumor suppression. DNA damage or activated oncogenes induces PUMA through p53 to promote apoptosis. Inflammatory cytokines induces PUMA through TNF-α to promote apoptosis. DNA damage, inflammatory cytokines and perhaps yet-to-be identified mediators and transcription factors (TF) might provoke PUMA-dependent apoptosis during inflammation.

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