Which can Attenuate Hepatotoxicity Induced By Pesticides Mixture Natural or Synthetic Phenolic Antioxidant

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Abstract: The present study examined the efficiency of green tea polyphenols as an example for natural polyphenols and butylated hydroxytoulene as an example for artificial polyphenols, in counteracting some of biochemical and histological alternations induced by repeated intoxication (28 days) with mixture of well known pesticides, widely investigated separetly. 6 groups of rats were treated as follows G1(control), G2 (p-mix , consists of, 1/60LD_{50} chloropyrifos =2mg/Kg b.wt, 1/200 LD_{50} of fenitrothion =2.5 mg/kgm b.wt and 1/100 LD_{50} of lambada cyhalothrin =0.17 mg/kg b.wt), G3(GT=100mg/animal), G4(p-mix+GT), G5(BHT=10mg/kgb.wt), G6(P-mix+BHT). Blood samples were taken at, 14 and 28 days for further biochemical parameters. Histopathological studies were carried out in liver tissue at the end of the experiment. Significant inhibition in plasma cholinesterase (ChE), damage in liver was observed and confirmed with elevation of plasma alanine aminotransferase (ALT), aspertate aminotransferase (AST) as well as elevation in oxidative stress (OS) marker malodialdehyde (MDA), plasma glucose, total cholesterol ,triglycerides and decrease in total glutathione content(GSH). In addition to angiogenic changes in blood vessels of animals treated with P-mix. Natural polyphenols (GT) supplemented to intoxicated rats induced pronounced counteracting effect in MDA, Glucose, cholesterol and triglycerides as well as promising effect in ALT&AST and liver tissue architecture and induce antiangiogenic effect. However, artificial polyphenols (BHT) supplementation has counteracting effect in MDA and GSH but it work synergistically with the p-mix on the other parameters. [Nature and Science. 2009;7(5):29-44]. (ISSN: 1545-0740).

Key Words: fenitrothion, chlorpyrifos, lambada cyhalothrin, mixture, polyphenols, green tea, butylated hydroxytoulene, oxidative stress, liver damage markers, angiogenesis

Introduction

Human and animal exposure to chemicals is rarely limited to a single chemical. Individuals are exposed daily to a variety of chemicals in food, drink, cosmetics and indoor and outdoor pollutants. In recent years, various environmental problems have led to increase concern about potential toxicity from exposure to multiple chemicals, including pesticide residues detected in food or water (Yang et al., 1989). Large-scale application of pesticides to crops and forests may contribute to the presence of toxic substances in the environment (John and Prakash, 2003). Pesticides are grouped into classes of compounds that have similar chemical structures and modes of toxic action. The most famous pesticide class is the organophosphate insecticides (OPs) organophosphorus (OPs) compounds are widely used in agriculture for vegetables and fruits protection, medicine and industry. Overspray of OPs resulting in serious damage to non-target species(Storm et al.,2000). Residual amounts of organophosphate (OP) pesticides have been detected in the soil, water bodies, vegetables, grains and other foods products (poet et al., 2004). Chlorpyrifos (CPF) and Fenitrothion, O, O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate, are organophosphorous insecticides, are now widely used for controlling a wide range of insects and pests. It was known that Ops cause acute toxicity through irreversible inhibition of acetylcholinesterase (ChE, EC 3.1.1.7), the enzyme responsible for the breakdown of the neurotransmitter acetylcholine. It has been reported that OPs may induce oxidative stress in humans (Almedia et al., 1997& Vidyasagar et al., 2004) and animals (Poovala et al., 1999 & verma 2001) when acutely exposed. On the other hand, it is well known that inhibition of acetylcholinesterase activity that is located in erythrocyte membranes can be an indicator of chronic toxicity of OPs. (Tinoco & Halperine 1998). Many other insecticide families also exhibit neurological activity and causes neurological damage, but at different target site as pyrethroids. Lambda-cyhalothrin is a broad-spectrum pyrethroid insecticide used to control a wide range of insect pests in a variety of crops. Lambda -cyhalothrin is highly used in the cotton plantation and in vegetable production (Leistra et al., 2003). Pyrethroids are potent sodium and potassium channel blockers that

produce subtle change in the channel's function, causing repetitive neural discharge (Soderlund et al., 2002). The mechanism by which pesticides cause damage involves multiple reaction pathways (Khan, 2006). Several studies of varying duration of exposure with organophosphorus or pyrethroid pesticides have postulated a possible role for the generation of free radicals and induction of oxidative stress (Tuzmen et al., 2008). In addition, it has been shown in previous studies that there is a correlation between acetylcholinesterase inhibition and lipid peroxidation levels in erythrocytes following subchronic and chronic exposure to OPs. (Akhgari et al., 2003 & Ranjbar et al., 2002). Other systems that could be affected by pesticides intoxication are immune system (Neishabouri et al., 2004) pancreas, liver and biochemical changes (Kalender et al., 2005). Polyphenols are our largest external source of antioxidants and are found in the plant foods that we eat. Polyphenols are naturally occurring chemicals and are responsible for the brightly colored pigments of many fruits and vegetables. Polyphenols have a significant antioxidant quality, by helping to protect tissues against oxidative stress (free radicals), certain polyphenols work as preventative medicines for problems such as cardiovascular diseases, cancer, arthritis, and auto immune disorders. Some have also exhibited anti-inflammatory and hepatoprotective effects. Polyphenols are secondary metabolites of plants and are widely distributed in plant derived foods such as cereals, legumes, nuts, vegetables, fruits and beverages such as green or black tea.(Bravo 1998). Polyphenols have been recently recognized as functionally active molecules, possessing antioxidant, anticancer rand antimutagenic properties (Chung et al., 1997). Tea polyphenols are the most significant group of green tea components, especially the catechin group of the flavonols. The major tea catechins are epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG), tea polyphenols possess a variety of biological functions, including antioxidant, anti-inflammatory, anticancer effects (Higdon & Frei 2003). Supplementation with black tea extract protects against free radical mediated oxidative stresss in hepatocytes of animals with pesticide mixture induced liver injury (Khan, 2006).Recently, tea polyphenols have been shown to protect against liver injury in animals intoxicated with chlorpyrifos insecticide (Khan & Kour 2007). Supplementation with (60mg/ animal) green tea polyphenols, partially attenuate oxidative stress resulted from the toxic effect of fenitrothion insecticide, on the liver and kidney of rats (Elhalwagy et al., 2008). The antiangiogenic property of green tea could be happened through multiple independent processes that include effects on gene expression signal processing or enzyme activities (Sogar et al., 2008). Phenolic antioxidant Butaylated hydroxytoluene (BHT), a preservative widely found in food as a food additive. Butylated hydroxytoluene (BHT) is known to inhibit tumor formation due to several chemical carcinogens including aflatoxin B1 (AFB1). The mechanism of action of BHT against AFB1 carcinogenesis is by induction of liver glutathione (GSH) S-transferases. As a result, the formation of AFB1-DNA binding is effectively inhibited (Allameh, 1997). Butylated hydroxytoluene (BHT) decreased the multiplicity of intestinal tumors (Balansky et al., 1992). Oral administration of BHT to rats also resulted in enhanced in vivo levels of GSH in lens, retina and cornea. In addition, a significant in vivo increase in the levels of GST, GSH-peroxidase, GSH-reductase, gamma-glutamylcysteine synthetase, and glucose 6-phosphate dehydrogenase was observed in the lens, retina, and cornea of BHT-fed rats (Ahmad et al., 1992). Fenirtrothion, chlorpyrifos and lambada cyhalothrin are well-known pesticides widely investigated separatly, and their effects on different organisms have been previously reported in separate studies. For this reason these pesticides were considered to be good model substances, relevant from the environmental perspective. On the other hand, we selected this kind of compounds because they are used in many tones annually in agriculture and horticulture and they are significant especially in greenhouse-based production of vegetables and fruits , In the present study, we investigated whether tea polyphenols or phenolic antioxidant (BHT) alleviate toxicity induced from mixture of the previous pesticides in rats.

Material &Methods

Animals

Male albino rats *Rattus norvegicus* (3–4) month's age, weighing between 150–180 g were used. Animals were supplied by the breeding unit of the Egyptian Organization for the Biology and Vaccine Production, Egypt. The animals were housed in plastic cages, fed *ad libitum* and allowed to adjust to the new environment for two weeks before starting the experiment. The rats were housed at 23 ± 2 ^{o}C dark/light cycle.

Chemicals:

Chlotpyrifos: pyriban (chlorpyrifos 48%EC) (O, O – Diethyl-O (3, 5, 6-trichloro-2-pyridyl phosphorothioat) was supplied by El Help company for pesticide industry- Egypt.

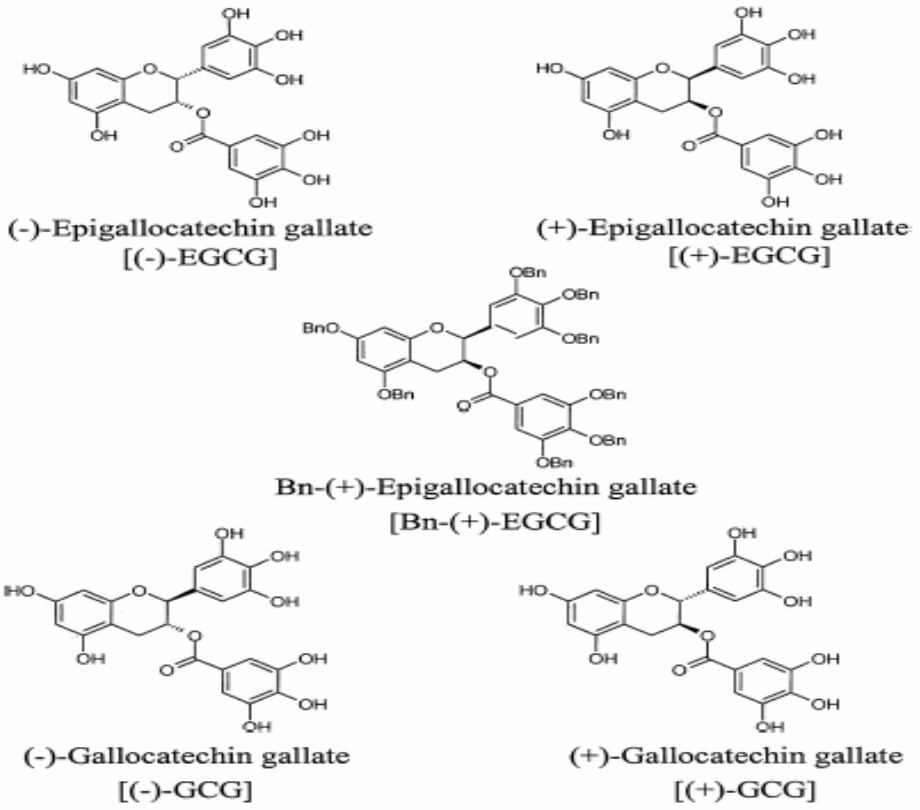
Fenitrothion: Sumithion (Fenitrothion 50% EC) (O,O-dimethyl O-4-nitro-m-tolyl phosphorothioate) was purchased from Kaffer Elzayat Co. for Insecticide Ind. Kaffr Elzayat, Egypt**.**

Lambada cyhalothrin: Karate (Lambda-cyhalothrin 2.5% EC) (cyano-3-phenoxybenzyl-3-(2-chloro-3, 3,3-trifluoro-1-propenyl)-2,2-dimethyl cyclopropanecarboxylate; is the most commonly and profusely used pyrethroid pesticide., was supplied by El Naser company for pesticide industry- Egypt.

Cocktail of combination of 3 types of pesticides were used in the present experiment

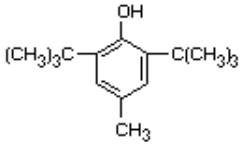
Antioxidant used:

Natural antioxidant: Green tea extract contains 98% polyphenols purchased from Hunan Changsha Yuanhang Biology Product Co., Ltd, China.contain a mixture of polyphenolic structures



Synthetic antioxidant:

Butylated hydroxytuleune (2:6-di-tert-butyl-p-cresol; 4-methyl-2:6-ditertiary-butylphenol) was purchased from Sigma Chemical Company (St. Louis, MO, USA).With chemical formula



Experimental Design:

All animals were treated according to the standard procedures laid down by OECD guidelines 407 (1992) repeated dose 28 days oral toxicity study in rodents. Animals were randomly divided into six experimental groups, five animals each as follows:

Group I (control): each animal in this group was given distilled water (1ml/animal) by gastric intubation every day for 28 days.

Group II (P-mix): rats were orally treated via gastric intubation with mixture of pesticides cocktail contain (1/60LD_{50} chloropyrifos =2mg/Kg b.wt, 1/200 LD_{50} of fenitrothion =2.5 mg/kgm b.wt and 1/100 LD_{50} of lambada cyhalothrin =0.17 mg/kg b.wt) every day for 28 consecutive days.

Group III(GT): rats were orally supplemented with 100mg /animal green tea extract for 28 days and served as(+ve control for GT) .

Group IIII (P-mix + GT): rats were orally supplemented with 100mg green tea/animal 1 hour after intoxication with pesticides mixture.

Group VII (BHT): rats were orally supplemented with 10mg /Kgm bwt butylated hydroxyl toluene for 28 days and served as(+ve control for BHT).

Group VIII (P-mix + BHT): rats were orally supplemented with 10mg/kgmbw butylated hydroxyl toulene 1 hour after intoxication with pesticides mixture.

Sampling

Blood samples collected from the retro-orbital plexus vein according to Schermer (1967). on heparinized tubes at 14 days and 28 days of treatment periods. Plasma samples were separated by centrifugation of the blood samples at 3600 rpm for 15 min. Plasma samples were kept at -20 C^{o} for subsequent use. At the end of the experiment, animals were dissected and samples of the liver were excised for histopathological studies.

Biochemical assay

Total reduced glutathione (GSH) was determined in erythrocytes by the method of Beutler et al. (1963) based on the development of a yellow color when DTNB is added to the supernatant of the precipitated RBCs containing sulfhydryl groups. Malondialdehyde (MDA) occurs as a result of lipid peroxidation in plasma and was measured according to Ohkawa et al. (1979) after incubation at 95 _C with thiobarbituric acid in aerobic conditions (pH 3.4). The pink color produced by these reactions was measured spectrophotometrically at 532 nm to measure malonaldehyde (MDA) levels. Plasma cholinesterase (ChE) was assayed by the method of Ellman et al. (1961). Markers for liver damage were determined using the commercial diagnostic kit of Stanbio Co., Spain. Plasma transaminases (AST and ALT) activities were determined according to Reitman and Frankel (1957). Cholesterol level was determined by the method of Henry(1974).Triglycerides were measured by the method of Schettler and Nussel(1975)**.** Plasma glucose level were determined according to Trinder (1959) using the commercial diagnostic kit of stanbio Co., Spain.

Histopathological Studies

Histopathological examination was carried out according to Drury and Wallington (1980). The liver tissue was dissected and the tissue samples were fixed in 10% formalin solution for 14–18 h, passed in a series of graded ethanol and embedded in paraffin. Paraffin sections were cut with at 5 µm thickness and stained with hematoxylin and eosin for light microscopic examination. The sections were examined and photographed on an Olympus light microscope (Olympus BX51, Tokyo, Japan) with attachment photograph machine (Olympus C-5050, Olympus Optical Co. Ltd., Japan). Identification of blood vessel areas for angiogenesis study, blood vessel areas was calculated by measurements of tissue boundaries and the area of all the blood vessels in the field this takes place by drowing a line around the blood vessel in each field, in one hot spot area. This was repeated in 5 fields by using the interactive morphometry software of the system on total magnification of (x100). The results appeared automatically on monitor in the form of the area of each blood vessel in each field measured in (2 µm) with total count and the area of all blood vessels in all the fields. The mean, the standard deviation, the minimum area, and the maximum area were measured (Niedergethmann et al., 2002)

Results

Effect of GT or BHT on Cholinesterase (ChE)

Inhibition in plasma acetyl cholinesterase (ChE) enzyme was noticed in P-mix intoxicated group significant as compared to the control at P<0.05 all through the experimental periods. However, animals supplemented with green tea polyphenols per se have a significant increase in ChE activity, it failed to counteract the effect of intoxication with P-mix. On the other hand, supplementation with BHT can not reduce the effect of p-mix intoxication but it work synergistically with p- mix to inhibit plasma ChE and this can be predicted from the effect of BHT per se as depicted in (Fig1).

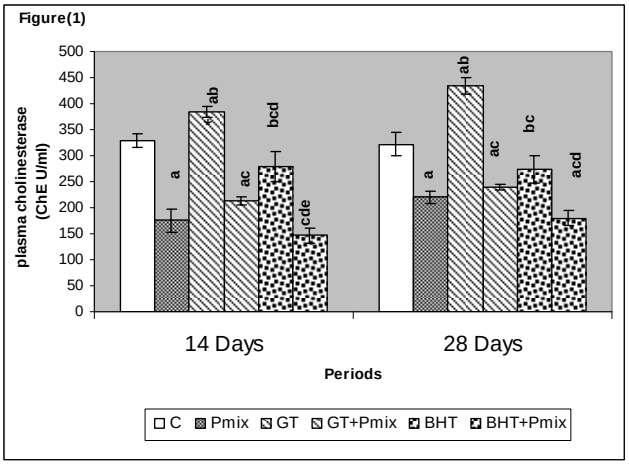


Figure (1): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on acetylcholinesterase (ChE) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE,[a] comparison of control and other groups($p < 0.05$), [b]comparison of pesticides mixture group and other groups($p < 0.05$),[c] comparison of green tea (+control) and other groups($p < 0.05$), [d] comparison of GT+ Pmix and other groups($p < 0.05$).

Effect of GT or BHT on Lipid peroxidation (MDA)

Intoxication with P-mix caused 1.5 fold increase in MDA level at 14& 28 days of treatment as compared to the control at P<0.05. Intoxicated rats supplemented with each of green tea polyphenols and BHT induced an early significant reduction in MDA level versus P-mix group to be reached nearly to the control level. However, slight reduction in MDA level was noticed at the end of the experiment significantly as compared to p-mix group (Fig 2).

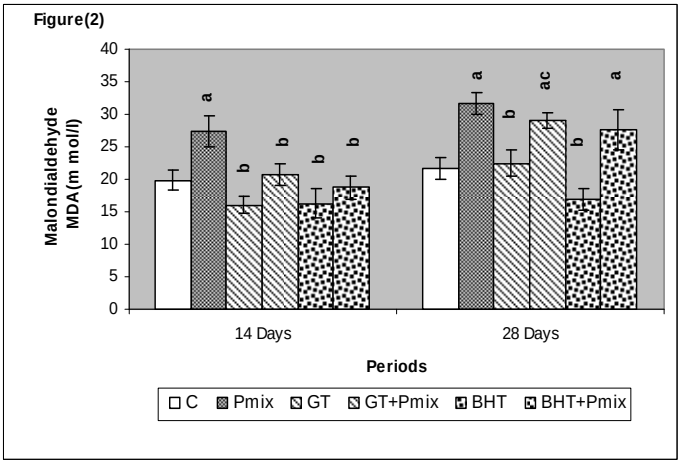


Figure (2): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on malondialdehyde (MDA) lipid peroxidation biomarker in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups ($p < 0.05$), comparison of pesticides mixture group and other groups ($p < 0.05$),[c] comparison of green tea (+control) and other groups($p < 0.05$), [d] comparison of GT+ Pmix and other groups($p < 0.05$)

Effect of GT or BHT on Total glutathione (GSH)

A pronounced reduction in blood GSH level was recorded in P-mix group at the end of the treatment as compared to the control. Supplementation with each of natural and synthetic phenolic compounds to intoxicated animals slightly improve the level of GSH at 14th and 28th days, this improvement was pronounced in BHT supplemented group as compared to the other groups at P< 0.05(Fig 3).

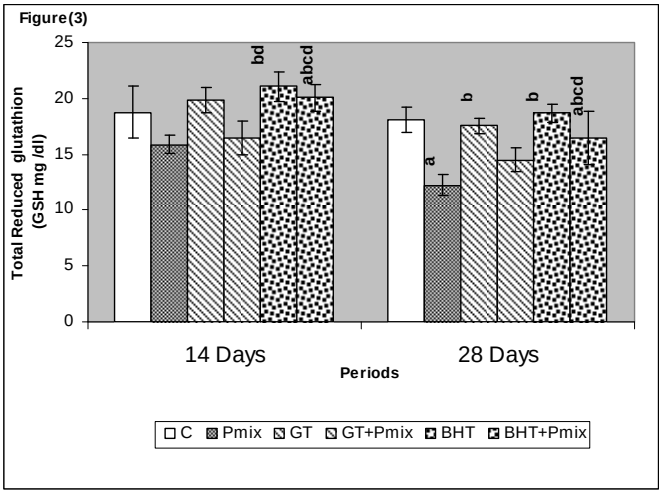


Figure (3): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) Total glutathione content (GSH) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups(p <0.05),[b] comparison of pesticides mixture group and other groups(p <0.05),[c] comparison of green tea (+control) and other groups(p <0.05), [d] comparison of GT+ Pmix and other groups(p <0.05).

Effect of GT or BHT on Liver Damage Markers

ALT and AST levels were found to be significantly (p<0.05) raised in p-mix intoxicated group as compared to the control, the increment in each parameter was 2 and 3 folds respectively. While supplementation with green tea per se not significantly affects activity of ALT & AST, it failed to improve their activities when supplemented to P-mix intoxicated group. However, BHT supplemented group have pronounced elevation in enzymes activities all through the experimental periods, this elevation is significant versus control and the respective non supplemented groups at p< 0.05(Figure 4&5).

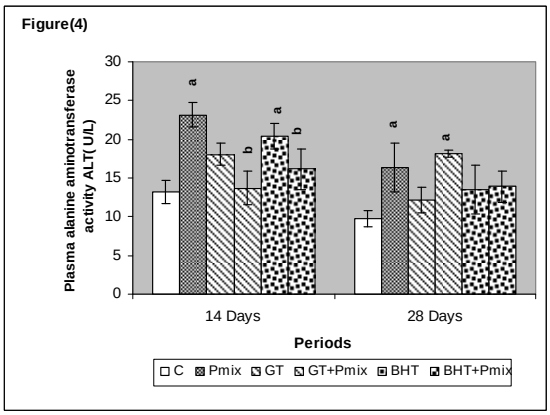


Figure (4): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on Alaninamino Transferase (ALT) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups (p<0.05), [b]comparison of pesticides mixture group and other groups (p<0.05),[c] comparison of green tea (+control) and other groups (p<0.05), [d] comparison of GT+ Pmix and other groups (p<0.05).

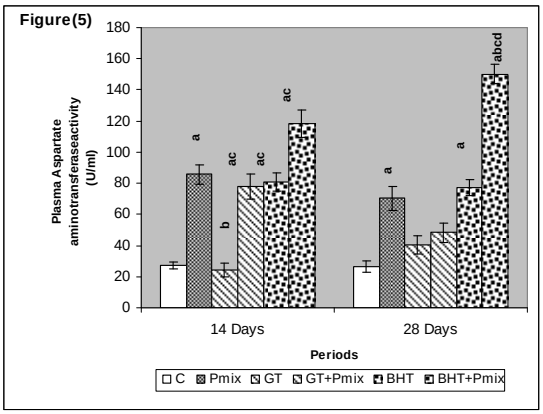


Figure (5): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) Aspartateamino Transferase (AST) in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups ($p<0.05$), [b]comparison of pesticides mixture group and other groups ($p<0.05$),[c] comparison of green tea (+control) and other groups ($p<0.05$), [d]comparison of GT+ Pmix and other groups ($p <0.05$).

Effect of GT or BHT on Plasma Glucose

A noticeable elevation in plasma glucose level was recorded in P-mix intoxicated animals, significant versus control at the end of 28th days of treatment. However administration of natural polyphenols (GT) attenuates this increment to be more or less near to the control level (Figure 6). On the other hands, intoxicated animals supplemented with artificial polyphenols (BHT) enhanced plasma glucose level to be elevated significantly versus control group and other treated groups, this effect must be attributed to the effect of BHT as demonstrated in group treated with BHT per se (Figure 6).

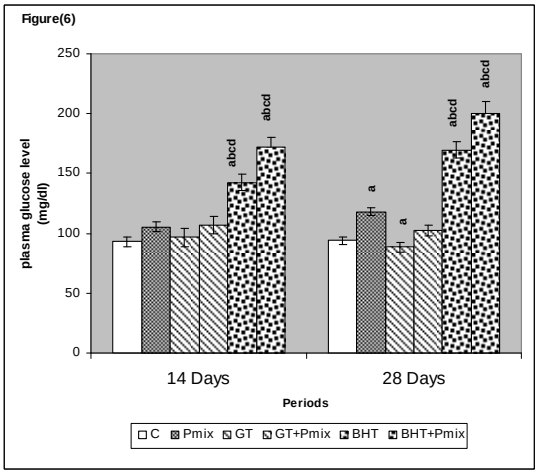


Figure (6): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on Glucose Level in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups ($p<0.05$), [b]comparison of pesticides mixture group and other groups ($p<0.05$),[c] comparison of green tea (+control) and other groups ($p<0.05$), [d] comparison of GT+ Pmix and other groups ($p<0.05$).

Effect of GT or BHT on Cholesterol and Triglycerides

As depicted in (Figure 7 & 8) treatment with P-mix induced significant increase in each of plasma cholesterol and triglycerides versus control $p< 0.05$. Supplementation with natural polyphenols (GT) counteracts p-mix effects a remarkable significant reduction in cholesterol level was recorded at 28th day in a counterpart (83.83±9.11 to 40.45±7.10 mg/dl, respectively). However, level of triglycerides more or less

nearly reached to the control level. On the other hand, supplementation with BHT failed to reduce the toxicity effect of p-mix. Significant increase in cholesterol and triglycerides level was recorded versus control and the respective non supplemented group at $p<0.05$.

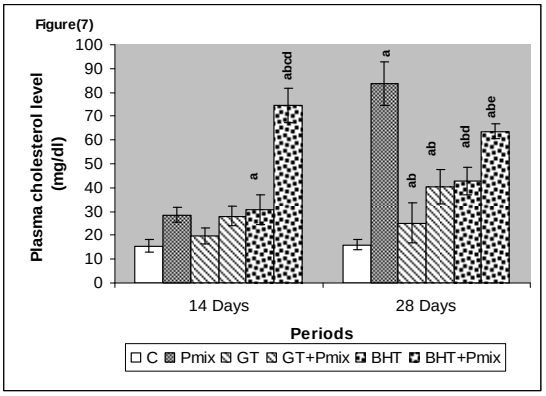


Figure (7): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) on total cholesterol in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups($p<0.05$), [b]comparison of pesticides mixture group and other groups($p<0.05$),[c] comparison of green tea (+control) and other groups($p<0.05$), [d] comparison of GT+ Pmix and other groups($p<0.05$).

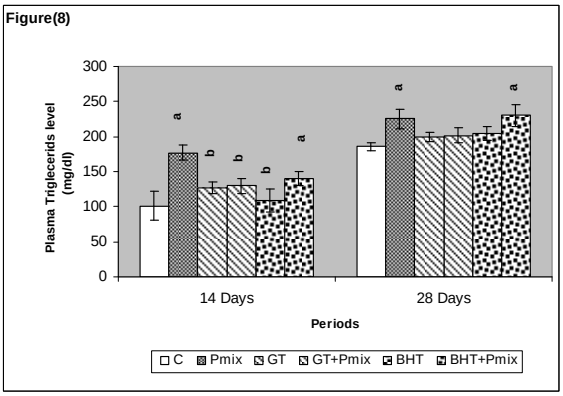


Figure (8): Effect of Green Tea polyphenols (GT) or Butylated hydroxytoluene (BHT) Triglecerides in plasma of rats intoxicated with mixture of pesticides. Data presented as mean+ SE, [a] comparison of control and other groups($p<0.05$), [b]comparison of pesticides mixture group and other groups($p<0.05$), [c]comparison of green tea (+control) and other groups($p<0.05$), [d] comparison of GT+ Pmix and other groups($p<0.05$).

Histopathological results

(Fig.9a) showed normal liver architecture with the central vein and radiating cords of normal hepatocytes with central rounded nuclei. Normal blood sinusoids appeared between the liver cords Liver section of rats administered orally with pesticides mixture for 28 days showing fatty liver and no assembly of the liver cells, depletion of their outer membranes with pyknotic nuclei (Fig. 9b). Liver section of rats supplemented orally with 100mg green tea extract/animal for 28 days per se and served as +ve control showing nearly normal structure of liver cells besides some of the fatty contents (Fig.9c). On the other hand, liver section of rats supplemented orally with 100mg green tea extract/animal 1 hour after intoxication with pesticides mixture for 28 days showing some central nuclei with undefined shapes and irregular cells with depletion in their cytoplasm, but the liver architecture was preserved that may consider some improvement but it needs more time in GT polyphenols supplementation (Fig 9d). Liver section of rat supplemented orally with 10mg/kg.b.wt. Butylated hydroxyl toluene (BHT) for 28 days showing more or less hepatic cells suffering from granulation and empty areas of their cytoplasm and irregular nuclei (Fig 9e). Liver section of rats supplemented orally with 10mg/kg.b.wt. Butylated hydroxyl toluene (BHT) and

pesticides mixture for 28 days showing depletion in the cytoplasm of hepatic cells with irregular nuclei (Fig 9f).

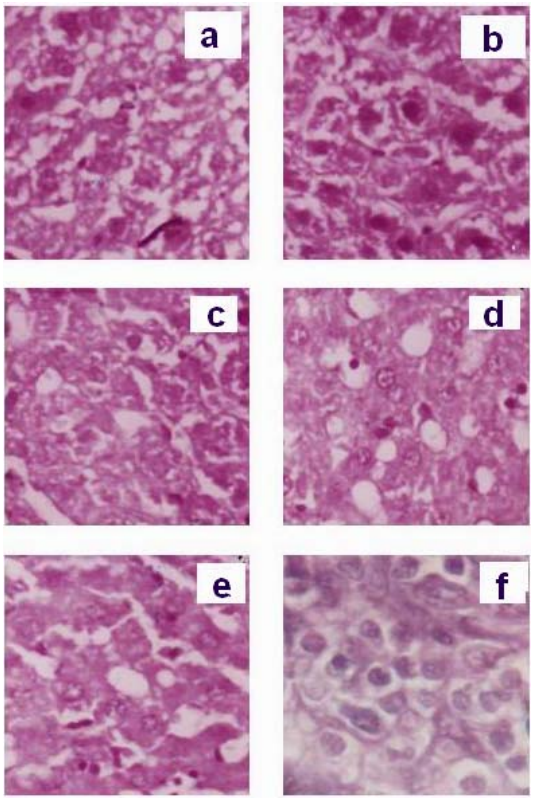


Fig. **(9):** Photomicrograph of the liver sections. Untreated control rat, showing a normal architecture of their hepatic cells (arrows) (**a**). Liver section of rat administered orally with pesticides mixture for 28 days showing fatty accumulation between the hepatic cells; depletion of the outer hepatic cell membrane with pyknosis of their nuclei (**b**) (arrows). Liver sections of rat supplemented with 100 mg green tea extract for 28 days showing normal of liver cells ©. Liver sections of rat supplemented with 100 mg green tea extract 1 hour after intoxication with the pesticide mixture for 28 days showing undefined nuclei and irregular cells with depletion of their nuclei (arrows) **(d**).Liver section of rat supplemented orally with 10mg/kg of butylated hyroxyl toluene (BHT) for 28 days showing more or less hepatic cells suffered from granulation and empty areas of their cytoplasm and irregular nuclei (arrows) **(e).** Liver sections of rat supplemented orally with 10mg/kg of BHT and intoxicated with the pesticide mixture for 28 days showing depletion of the cytoplasm of hepatic cells with irregular nuclei (arrows)**(f).** All photomicrographs stained with H & E 400X**.**

Immunohistological (Antiangiogenesis) results

As expressed in table 1 and fig. 10a which showed the liver sections of untreated rat, abundance blood vessels and hepatic cells immunolabelled with monoclonal antibody directed against the Factor-VIII-associated antigen. Liver section of rats administered orally with pesticides mixture for 28 days showing decrease in the invading blood vessels between hepatic cells. The results of measured areas of blood vessels in the hot spot areas showed decrease (35.9±6.88) in the blood vessel areas compared to control group (Table 1 and fig.10b). Liver section of rat supplemented orally with 100mg green tea extract/animal for 28 days showing nearly normal invading blood vessels and hepatic sinuses. The measured values showed significant increase in the values (141.99 ± 39.44), table 1 and fig10c. Liver section of rats supplemented orally with 100mg green tea extract/animal 1 hour after intoxication with pesticides mixture for 28 days showing an increase in the blood vessels. The measured values obtained non significant increase (79.28 ± 18.14) compared to the control group (Table 1 and fig.10d). Liver section of rat supplemented orally with 10mg/k.b.wt. butylated hydroxyl toluene (BHT) for 28 days showing that some enlargement in the blood vessels between hepatic cells. The values showed no significant increase (67.76 ± 12.06), table 1 and fig.10e. Liver section of rats supplemented orally with 10mg/k.b.wt. butylated hydroxyl toluene (BHT) and

pesticides mixture for 28 days showing some constrictions of blood vessels and hepatic sinuses. Significant increase was noticed in the measured blood vessels areas (61.65 ± 17.61) compared to control group (Table 1 and fig.10 f).

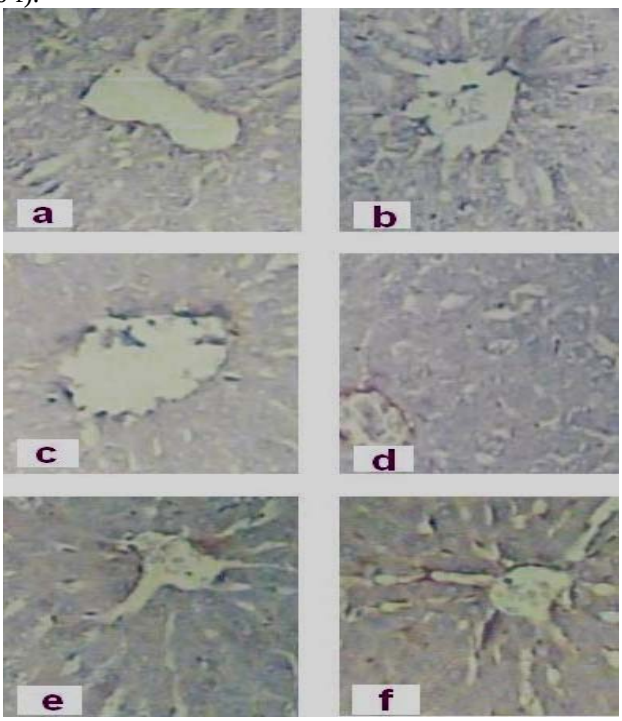


Fig (10): Photomicrograph of the liver section of untreated rat showing abundance blood vessels between the hepatic cells immunolabelled with monoclonal antibody against the factor – VIII associated antigen **(a)**. Liver section of rat administered orally with pesticide mixture for 28 days showing decrease in the invading hepatic vessels immunolabelled with monoclonal antibody against the factor – VIII associated antigen **(b).** Liver section of rat supplemented orally with 100mg/kg of green tea extract for 28 days showing nearly normal blood vessels and hepatic sinusoids immunolabelled with monoclonal antibody against the factor – VIII associated antigen **(c).** Liver section of rat supplemented orally with 100mg/kg of green tea extract 1 hour after intoxication with pesticide mixture for 28 days immunolabelled with monoclonal antibody against the factor – VIII associated antigen showing an increase in the blood vessels growing **(d).** Liver section of rat supplemented orally with 10mg/kg of butylated hydroxyl toluene for 28 days immunolabelled with monoclonal antibody against the factor – VIII associated antigen showing some enlargement of the diameters of the hepatic vessels **(e).** Liver section of rat supplemented orally with 10mg/kg of butylated hydroxyl toluene BHT 1 hour after intoxication of pesticide mixture for 28 days immunolabelled with monoclonal antibody against the factor – VIII associated antigen showing constrictions between the hepatic vessels **(f).**

Table (1): The Angiogenesis effects of Green Tea polyphenols and BHT polyphenols on the toxicity of pesticides mixture

Groups	control	P-mix	GT (+vecontrol)	Pmix +GT	BHT (+vecontrol)	P-mix +BHT
Mean + SE	55.8 + 7.42	35.92 + 6.88*	141.99 + 39.33*	79.28 + 18.14	67.76 + 12.06	61.65 + 17.61*
Max Area	120.8	100.72	553.07	293.86	261.43	317.91
Mini Area	9.8	12.02	14.7	17.56	17.59	11.74

Discussion

Several studies had been conducted to investigate the adverse effects induced as a result of individual exposure to different pesticides. Fenitrothion, lambada cyhalothrin and chlorpyrifos insecticides included in our examined mixture. They have been examined individually in previous studies by (El-Halwagy et al., 2008 , El-Demerdash 2007). Substantial information is available regarding their environmental and ecological impact , not much is known in regard to the mixture toxicity in mammalian system . The mechanism by which pesticide cause damage varied according the structure of the pesticides, the primary mechanism of action and most acutely life threaten effect of Ops insecticides are related to the accumulation of acetylcholine within the cholinergic synapses resulting from inhibition of acetyl cholinesterase by active oxon metabolites (Mileson et al., 1998). The main effects of pyrethroids are on sodium and chloride channels, pyrethroid modify the gating characteristics of voltage sensitive sodium channels to delay their closure (Bradberry et al., 2005). Increasing Na+ influx into synaptic terminals and creating a hypopolarized hyper irritable synaptic membrane, which in turn increases the release of the neurotransmitter acetylcholine (Bandettini et al., 1992 and Rao & Rao, 1993). Previous facts explain the remarkable inhibition in plasma ChE induced when animals treated with cocktail (Fn+CPF +lambada cyhalothrin) mixture , these results are coincide with that recorded by Latuszynska et al., 2001 and 2003; chlorpyrifos and cypermethrin administrated in a mixture strongly inhibited cholinesterase activity in plasma, this inhibition was associated with the effect of chlorpyrifos. An increase in the amount of dissolved oxygen and reactive oxygen species (ROS) in the blood and excessive generation of highly reactive oxidants results in tissue damage, called as oxidative stress. ROS are derived from a variety of sources, such as the xanthine oxidase system, activated neutrophils, the electron transport chain of mitochondria, and the arachidonic acid pathway. Since free radicals have very short half-lives, the clinical assessment of oxidative stress in vivo is based on the measurement of different stable oxidized products of modified lipids, proteins, carbohydrates and nucleic acids. Malonyldialdehyde (MDA), is one of the most widely used biomarkers of oxidative stress, is produced enzymatically by the breakdown of unstable hydro peroxides during per oxidation of unsaturated fatty acylmoietie (Roberts and Morrow 2000). Our results demonstrated a significant increase in plasma MDA level after intoxication with cocktaile of pesticides for 28 days. It is plausible to speculate from our results that (CPF, Fn and lambada cyhalothrin) treatment may result in peroxidation of polyunsaturated fatty acids , leading to degradiation of phospholipids and ultimately result in cellular deterioration (Tappel.1973), science coktaile of investigated pesticides are lipophelic substances, they may interacting with the cellular plasma membrane (Hazarika et al.,2003). Depletion in total glutathione content GSH resulted from intoxication with pesticides mixture and concurrent to the elevation of lipid peroxidation biomarker MDA. Reduced glutathione plays an important role in the detoxification of xenobiotic and antioxidation of reactive oxygen species and free radicals by oxidation of GSH to glutathione disulfide (GSSG) so increasing in oxidative stress accompanied by decline of GSH as reported by (Manna et al., 2005). The extent of liver damage appears to be considerable as evidenced by the increase in plasma levels of ALT & AST as shown in Figure (5&6) resulted from intoxication with cocktail of pesticides, these results are in content of the previous results recorded by (Elhalwagy et al.,2008, Khan,2006 and Muthuviveganandavel et al., 2008) oral intoxication with each of fenitrothion ,chlorpyrifos and cypermethrin ,respectively, induced elevation in ALT & AST activities as a result of liver tissues damage expressed by histological examination and run parallel with marked histological alterations were observed in the liver of rats treated with pesticide mixture for 28 days in our study in which tissue disorganized, cytoplasmic vacuolization (fatty degeneration), cellular necrosis and congestion of blood vessels. One of the characteristics of pesticides is induction of stress, stress is a response to every situations which threatening homeostasis and result in activation of hypothalamic pituitary adrenal (APA) axis and sympathetic autonomic nervous system, which consequently lead to hyperglycemia, (Mechanick, 2006). Stimulation of sympathetic nervous system during stress leads to enhanced release of catecholamines, glucagon and growth hormone which result in promotion of gluconeogenesis, glycogenolysis, insulin resistance and constitution of hyprerglycemia. Also, Ayub shah & Gupta (1997) & Husain et al., (1994) recorded an elevation in glucose level as a result of permethrin or deltamethrin intoxication mainwhile, (Rahimi and Abdollahi 2007) reported significant elevation in glucose level after OPs intoxication, these facts explain significant elevation in plasma glucose as a result of pesticides mixture intoxication recorded in the present study. Our data also, pin point the role of pesticides mixture on the elevation of the level of plasma total cholesterol and triglycerides, the increase in the level of serum cholesterol may be due to increase synthesis of serum cholesterol in the liver (Enan et al., 1987)

or may be attributed to an inhibition of lipase lipoproteins (Goldberg et al., 1982). Each of artificial or natural polyphenols supplementation to pesticides mixture intoxicated animals, failed to counteract the inhibition in plasma ChE induced by pesticides cocktail intoxication. Catechins one of green tea polyphenols reacts with peroxyl radicals in phospholipids bilayers via a single electron transfer followed by deprotonation prevent inhibition in AchE enzyme (Javanovic et al., 1996), these findings explain the significant increase in ChE enzyme activity in plasma of animals supplemented with green tea polyphenols and served as positive control. However, artificial polyphenols BHT works synergistically with the inhibitory effect of pesticides mixture intoxication on plasma ChE. It must be noted here that BHT per se has an inhibitory effect on plasma ChE as depicted in previous study reported by (Bilusic et al., 2008). With respect to the effect of polyphenols supplementation on lipid peroxidation biomarker (MDA) each of BHT & green tea polyphenols try to counteract the increase in MDA resulted from P-mix intoxication to be more or less near the control level. Green tea polyphenols do its effect on lipid per oxidation by enhancing the level of antioxidant directly of spare the endogenous pool of GSH from being exhausted by generated free radicals (Skrzydlewska et al., 2002& Kane 2006). A molecule of BHT is able to react with 2 peroxyl radicals to yield products that bare more stable (Black 2004) as well as it enhance the level of GSH (Ahmad et al.,1992). The previous findings run parallel with our findings in GSH results. green tea obtained ameliorating affect in the liver section of rats with some fatty degeneration. (Zhen et al., 2007) recorded that EGCG content of green tea arrested progression of hepatic fibrosis. (Feng et al., 2002) reported that tea flavines could prevent cellular DNA damage by inhibiting oxidative stress. Also, (Zhong et al., 2003), reported that polyphenols of green tea scavenge oxygen radicals and prevent activation of stellate cells minimizing liver fibrosis. green tea polyphenols needs more time to ameliorate the toxic effect of pesticides on ALT & AST (Elhalwagy et al., 2008) . Significant reducation in cholesterol and triglycerids were observed in green tea polyphenols supplemented groups, this obtained results are in consistant in previous studies (Matsumoto et al., 1998). In contrast, BHT failed to counteract the elevation in plasma cholesterol and induced triglycerides , these results are coincide with that obtained by (Faine et al.,2006) In spite of improvement on the oxidative stress parameters , an enhancement in the liver enzymes biomarkers were recorded in BHT polyphenols supplemented groups permeabilized the plasma and mitochondrial membranes to enzymes leakage accompanied by enhanced membrane fluidity in animals treated with BHT (Shertezer et al., 1991). BHT is an inducer of cytochrome P450 in the hepatocytes (Price et al., 2008), it was recorded that BHT was a multiple mechanisms especially cytotoxic effect through interactions with neutrophils membranes and the ROS scavenging effect (Kobeya et al., 2008). However, (Guarisco et al., 2008) showed that BHT is capable to induce oxidative and metabolic alterations similarly to some pathological disorders. Vessel formation occurs mainly through two sequential mechanisms (Carmeliet, 2000). De novo formation of blood vessels during embryonic development is called vasculogenesis. Mesoderm-derived stem cells (hemangioblasts) form aggregates (blood islands), and they develop into primitive hematopoietic and endothelial cells (angioblasts). Angioblasts differentiate and proliferate in situ to form a primitive network. On the other hand, the formation of new capillaries from preexisting vessels is
called angiogenesis. The principle mechanism of vessel formation in adults is angiogenesis.

As regards to the antiangiogenic results revealed nearly normal invading blood vessels and hepatic sinuses. (Jung et al., 2001) and (Zhang et al., 2006) recorded that EGCG of green tea inhibited the increase of VEGF expression, blocking its induction and leading to the antiangiogenesic effect of green tea. Other investigation by inhibiting metalloproteins and the vascular endothelial growth factor.

In conclusion, exposure to pesticides mixtures showed highly degeneration of hepatic cells and the antiangiogenic effect decrease in the invading blood vessels between hepatic cells as well as disturbance in different biochemical parameters. Supplementation with natural polyphenols as green tea showed some ameliorating effects but may be needs more time for the present investigated dose and another doses can be investigated . However, artificial polyphenols BHT increases the incidence of degeneration of hepatic cells. Although, there is an improvement in oxidative stress parameters and enhancement of GSH level.

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