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The protective efficacy of propolis against multi heavy metals-induced oxidative stress and hepato-renal damage in the males of albino rats

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Abstract

Lead (Pb), Nickel (Ni), Cadmium (Cd), and Antimony (Sb) are toxic metals which are capable of accumulating within vital organs of both humans and animals, inducing reactive oxygen species (ROS) production and causing severe health hazards within their biological systems. The study has been designed to clarify the hepato-renal protective effects of propolis extract against toxic metals mixture via oral administration to the males of albino rats. The concentrations of Pb, Cd, Ni and Sb as well as the activities of glutathione peroxidase (Gpx), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) were all determined in the tissues of liver and kidney; while aspartate transaminase (ASAT), alanine transaminase (ALAT), total protein (TP), urea and createnine, were measured in the serum of experimental rats, besides, histopathological examinations in the tissues of liver and kidney. Metals mixture oral administration resulted in a significant increase in the concentrations of Pb, Cd, Ni and Sb within the tissues of liver and kidney of all rats from experimental groups accompanied by, a significant depletion in the levels of Gpx, GR, CAT and SOD in the same tissues. Moreover, a statistical elevation in the levels of ALAT, ASAT and urea were observed in the serum of rats from the same groups as well as, some histopathological alterations in the tissues of liver and kidney. The oral administration of propolis provided a significantly therapeutic role against metals mixture-induced hepato-renal toxicity with relative improving to histopathological changes because of its anti-oxidative action and the potentiality to nullify the deleterious effects of free radicals through reactivating the normal defence mechanism of endogenous enzymes as concluded from the present investigation.

Key words: heavy metals, propolis, hepato-renal, antioxidants, oxidative stress, biochemical, histopathology.

1. Introduction

Heavy metals are well known for many years to be; toxic, affecting the internal organs of the human body and harm public health even at trace levels **[1].** They can disrupt membrane potentials of some normal cell and tissue functions through binding with proteins and other bio-molecules **[2].** Metals cannot be decomposed biologically, instead, they transform from one oxidation state or organic complex to another **[3].** Among the various heavy metals, lead, cadmium, nickel and antimony are proverbial as the most dangerous metals due to their high toxicity that attributed primarily to oxidative stress which is one of the critical mechanisms implicated in the heavy metals-induced toxicity since, the free radicals in biological systems produced from redox reactions carried out by certain metals, consequently, these reactions create oxidative damage to proteins and DNA and inhibit the antioxidant defense systems **[4, 5].** Because oxidative damages in various body organs are correlated with toxic metals exposure, great attention was given for the usage of natural products to strengthen the cell antioxidant and to protect it from the heavy metals-induced damage **[6].** Natural antioxidants are safe, effective and affordable agents when compared to other therapeutic agents, such characteristics make them an excellent choice in the prevention and treatment of toxicities. Active constituents from natural origins including; curcumin, flavonoids, vitamin E, β–Carotene, *Nigella Sativa* (black seed), Gum Arabic, *Physalis peruviana*, Naringenin and propolis possess the ability to treat or alleviate toxicities in the various body systems which may be induced by heavy metals. Moreover, the antioxidant properties of these natural products have an ability to reduce the toxic effect produced by reactive oxygen species such as hydrogen peroxide and hydroperoxides that lead to a depletion in the antioxidant defense mechanism of the body inducing a lipid peroxidation along with a cell membrane

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disruption and nucleic acids oxidation and cell damage at the end **[7, 8]**. The main and most popular honeybee product is honey which has many beneficial therapeutic effects such as antibacterial, antiinflammatory, hepatoprotective, antioxidant, and antihypertensive effects. However, there are other bee products which are secreted either alone or variously mixed including; royal jelly, beeswax, propolis, bee pollen, and bee venom; all of them have been used by humans for their several biological and curative properties. Honey has been used for cicatrization problems and diabetes, royal jelly for diabetes and rheumatoid arthritis, propolis for disinfection and gingivitis, bee venom for Parkinsons' and rheumatoid arthritis, bee pollen for prostatitis, stomach ulcers and infectious diseases, whereas, bees wax plays a role as a thickener, binder, drug carrier and release retardant **[9 - 11].**

Propolis is a natural sticky, resinous mixture produced by honey bees and gathered from the tree buds, leaves, sap, flows, and other botanical sources **[12].** It has been characterized variously as an antibacterial, antiviral, antiinflammatory, antioxidant, and anticarcinogenesis agent **[13].** Propolis consists mainly from flavonoids, phenolics and other various bioactive compounds like caffeic acid phenethyl ester (CAPE) which has hepato-renal protective effects against the cytotoxic injuries **[14]**. Moreover, the bio-flavanoids of propolis are antioxidant molecules that play very important role in the scavenging of free radicals **[15, 16].** Propolis works through various mechanisms and at various sites including chelation or antagonism of heavy metals in order to, nullifying their capacity to generate free radicals, thereby neutralizing their oxidative effect and interrupting the auto-oxidative chain reaction **[17].** Propolis extract reduces the activity of activated macrophages and the expression of the matrix metalloproteinase-9 (MMP-9) gene in a dose-dependent manner **[18].** Liver and kidney are important organs when the effects of toxins are investigated, since these organs play a central role in the metabolism and detoxification of biological substances. Also, most substances absorbed by the intestine pass first through liver or kidney where toxins and heavy metals may accumulate **[19].** Hence, there is little information about the hepato-renal protection of propolis against multi-heavy metals toxicity. Therefore, the purpose of this experimental study is providing a new picture to the propolis for better understanding of its hepato-renal protective effects against ROS radicals caused by metals oral mixture administration that inhibit the normal defense mechanism of endogenous enzymes.

2. Experimental details

Material and Methods

Chemicals:

All reagents and chemicals were of analytical grade quality with high purity. Lead, cadmium, nickel and antimony (SCP SCIENCE, Canada) were used for mixture preparation of oral administration solutions. Standard solutions (Merck, Germany) were used to create calibration curves for toxic metals analysis, while concentrated HNO_3 (65%, Merck, Germany) and H_2O_2 (30%, Sigma-Aldrich, Germany) were used for tissue digestion. All chemicals and reagents for the examination of antioxidant status were purchased from Bio-diagnostic (Egypt).

Propolis:

Propolis was obtained from hives of royal bee company Cairo, Egypt during summer 2018. It was bulk of glue like brownish material resulted from scrapping off the frames of bee hives. It was initially stored in a freezer in order to kill insect eggs and to facilitate removal of debris and fragmentation. Propolis extract was performed using Ethanol Extracts of Propolis [EEP] by using 250g of propolis powder which transferred to 1000 ml volumetric flask, and completed to 1000 ml by 70% ethanol HPLC grade in the absence of bright light, with moderate shaking using a magnet stirrer for 1 day at room temperature. After a week, the extracts were filtered through 0.45 µm filter paper using vacuum filtration, and the solvent was evaporated off in a vacuum oven at a temperature of 60˚C to obtain pure propolis extracts which diluted by saline and given to rats **[20]**. Ethanol 70% produces a higher yield values than other solvents, it was found to extract most of the active components of propolis. The yield of propolis from ethanol 70% was about 18% according to The yield of extraction which was calculated using the following formula: **Yield % = (Pe / Pm) × 100**, Where: Pe is weight of propolis extract (g) and Pm is weight of raw propolis (g)

Animals:

Thirty male *Wistar rats* weighing approximately 200 g ± 10 g were purchased from The Egyptian Holding Company for Biological Products & Vaccines, Cairo, Egypt and used as experimental animals. Rats were housed and maintained under standard controlled conditions of good ventilation, normal temperatures, and humidity range (temperature 25 ±4 °C, relative humidity of 35% to 60%, 12-h light-dark cycle). They were allowed free access to drinking water (metal-free water) unlike, feeding which was restricted to be introduced by limited amount (50 g) once daily for each group. During the experiment

period (75 days), the feeding time was one hour before dosing of metals mixture (Pb, Ni, Cd, Sb), while propolis dosing was after two hours of metals administration. The experiment was carried out according to the Guide for the care and use of Laboratory Animals published by the National Institutes of Health (No. 85:23, 1996) and in compliance with the principles and guidelines of the Scientific Research Ethics Committee, Faculty of Science Al-Azhar University under Certificate Reference Number AZHAR11/2017. All rats underwent to good care and minimum pain suffering during the experiment, besides, anaesthetising before blood collection and dissection processes.

Study design and experimental procedure:

After ten days of acclimatization, thirty rats were placed into suitable cages by ratio of ten rats per cage. Using physical randomization; three tens of colored sticky papers (Red, Blue, Green) were numbered from 0 - 9 per each color, then the three colors were identified to three experimental groups one negative control group (Red) and two experimental groups; heavy metals group (Blue) and propolis group (Green). The first rat was assigned to the Red group, the next rat to the blue group, the next to the Green and so on. The study was single-blind a long 75 days where, experimental groups received a single dose (1 ml/rat/day) of freshly prepared aqueous solution containing heavy metals mixture with concentrations exceeds the maximum limits of both WHO and Egyptian standard regulation by 10 fold which are; (100, 200, 30, and 200) ppb for (lead, nickel, cadmium, and antimony), respectively **21 – 24]**. The propolis group was post-treated by propolis extract in a dose 200 mg/kg/day body weight (b.w.) for 75 days while the control group was neither treated nor contaminated. Dosing of all animals was performed by using an oral gavage tube directly into stomach.

Samples Preparations:

After 75 days of metals mixture intoxication and exactly at day 76, blood samples were collected where retro-orbital venous plexus exist, after anesthetic administration. Suitable amounts of blood were collected in test tubes without anticoagulant for obtaining serum which was separated and transferred to eppendorf tubes to be frozen for biochemical assays. At the same day, all rats were dissected then livers and kidneys were removed and separated into three parts. One tissue sample (0.5 g) was frozen and stored for the investigation of antioxidant status; a second was frozen and stored for toxic metals analysis and a third tissue sample was preserved in formalin for histopathological examination.

Toxic Metals Analysis:

After the animals had been sacrificed, wet tissue samples of livers and kidneys weighing about 0.5 g were placed in Teflon containers with 9 ml conc. HNO_3 and 1 ml H_2O_2 and digested using high performance microwave sample digestion (model Milestone ETHOS UP). Digestion was carried out according to the Milestone's recommendations **[25]**. After digestion program, the samples were transferred to 50 ml volumetric flasks and the volumes were completed to 50 ml using free-metal water (grade A). The amounts of Pb, Ni, Cd, and Sb in the tissue samples were determined by Inductively Coupled Plasma Optical Emission spectroscopy ICP-OES (I Cap Thermo 7400, Thermo Fisher Scientific, Waltham, Ma, USA) **[26]**.

Biochemical Assays:

The measured biochemical parameters in rat's serum included total protein (TP), alanine aminotransferase enzyme activity (ALAT), aspartate aminotransferase enzyme activity (ASAT), serum urea and serum creatinine. All biochemical assays were performed with commercial reagents and according to controlled working instructions of Roche Cobas device owned to Mabaret El-Asafra Labs. Alexandria, Egypt. Besides, they were confirmed using UV-VIS spectrophotometer (Labomed, Inc; Los Angeles, USA) methods; TP **[27],** ALAT & ASAT **28],** urea **[29]** and createnine 30**]**.

Antioxidants Analysis:

Tissues samples (livers & kidneys) were rapidly excised, washed in ice-cold 0.9% NaCl, then an exact weight of each organ (0.5 g) was grinded through homogenizer in 4 ml saline solution (NaCl 0.9%). Each sample was centrifuged at 4000 RPM for 15 minutes then the obtained supernatant was transferred into eppendorf tubes, and frozen to be analyzed for antioxidants biomarker. The activities of antioxidants enzymes were measured according to references methods including; glutathione peroxidase (Gpx)**[31]**, glutathione reductase (GR) **[32]**, catalase (CAT) **[33]**, and superoxide dismutase (SOD) **[34]**.

Histopathological Analysis

Liver and kidney tissues were subjected to histopathological examination. Microscopic examinations on paraffin embedded 5 µm tissue sections with hematoxylin-eosin were performed. Each section was examined under an optical microscope.

Statistical Analysis

Statistical analysis was performed using Graphpad prism 6.0 statistics software (Graphpad Inc. San Diego, CA, USA). One-way and two-way analysis of variance (ANOVA) test were used followed by Tukey's

multiple comparisons test. p -Values less than 0.05 were considered significant.

3. Results

Metals concentrations in liver and kidney:

Both of experimental groups, (heavy metals group and propolis group) receiving a single dose (1 ml/ 200g. rat/day) of the heavy metals mixture demonstrated significantly higher levels of Pb, Ni, Cd, and Sb in the liver when compared to the control group. The measured levels of metals in the liver of experimental groups which received toxic mixture exhibited a statistically high significant difference when compared to the control ($p < 0.0001$). Additionally, the kidneys tissues of the heavy metals group showed statistically higher significant ($p < 0.0001$) in the levels of Ni whereas, Pb, Cd, and Sb were also significant ($p < 0.001$) when compared to the control group. The amount of toxic metals in the heavy metals group had higher concentrations in comparison with values in the control and propolis groups. Furthermore, treatment with propolis revealed remarkable reduction in the concentrations of Pb, Ni, Cd, and Sb in both, liver and kidney tissues when compared with positive control group (heavy metals group). At the same time, liver exhibited high susceptibility to accumulate Pb and Sb while kidney was more affected by Ni and Cd. The deposition order of metals was Ni > Pb > Sb > Cd in the liver and Ni > Cd > Sb > Pb in the kidney, (Table, 1).

Biochemical Assasy:

Acute exposure to the investigated toxic metals administered in a mixture form resulted in the altered profile of some biochemical parameters. Both experimental groups had higher concentrations of ALAT, ASAT and urea compared to the control group, while there was a trivial increase in creatinine levels in heavy metals groups, unlike total protein which showed slight decrease levels in both experimental groups, there were statistically significant differences in the levels of ALAT, ASAT, and urea among heavy metals group, when compared to the controls ($p < 0.001$, $p < 0.01$, $p < 0.01$, respectively), while total protein and createnine levels didn't produce any differences in the same groups when compared to the control. However, urea and createnine showed significant difference when compared with the heavy metals group ($p < 0.01$, $p < 0.05$). Biochemical parameters are shown in Table (2).

Table, (1): Concentrations of toxic metals (µg/g wet wt. ± SE) in livers and kidneys of different groups.

Groups	Metals / Organs	Lead (Pb)	Nickel (Ni)	Cadmium (Cd)	Antimony (Sb)
Control	Liver	0.23 ± 0.05	20.77 ± 0.22	1.48 ± 0.09	0.5 ± 0.13
	Kidney	1.43 ± 0.1	105.1 ± 9.27	6.41 ± 0.67	5.17 ± 1.17
Heavy metals	Liver	179.07 ± 8.92 ****	1176.16 ± 24.52 ****	56.74 ± 4.19 ****	144.34 ± 12.04 ****
	Kidney	47.4 ± 3.92 ***	1829.59 ± 41.55 ****	103.05 ± 5.54 ***	53.06 ± 7.51 ***
Heavy metals +Propolis	Liver	^^^^ 33.56 ± 6.64 **	^^^ 532.28 ± 12.73 ***	^^^^ 49.36 ± 1.39 **	^^^^ 29.07 ± 7.63 **
	Kidney	^^ 17.36 ± 2.34	^^^^ 1014.54 ± 132.45 ****	39.04 ± 7.08 ***	^^^ 20.5 ± 2.55 *

Observed metals are expressed on wet tissues. Statistically significant differences *($p < 0.05$)* compared to control group are indicated by *, while those compared to heavy metals group are indicated by ^. Statistical evaluation was performed using one-way ANOVA followed by Tukey's multiple comparisons test. * ^ *$p < 0.05$;* ** ^^ *$p < 0.01$;* *** ^^^ *$p < 0.001$;* **** ^^^^ *$p < 0.0001$*.

Antioxidants Status:

After exposure to the heavy metals mixture, the activities of all investigated antioxidant enzymes (GPx, GR, SOD, CAT) exhibited a downward trend in liver and kidney tissues among both experimental groups (Heavy metals group and propolis group) with statistically significant effects when compared to the control group. Administration of propolis treatment demonstrated remarkable restoring in the activities of GPx, GR, SOD and CAT with statistically significant elevations ($p < 0.001$, $p < 0.0001$, $p < 0.0001$, $p < 0.01$, respectively) in the liver and ($p < 0.01$, $p < 0.01$, $p < 0.001$, $p < 0.01$) in the kidney when compared to heavy metals group. Observed redox parameters in livers and kidneys of rats are presented in Table (3).

Table (2): Levels of some Biochemical parameters in the serum of rats at the experimental groups.

Parameters / Groups	ALAT U/l	ASAT U/l	Total Protein g/dl	Urea mg/dl	Creatinine mg/dl
Control	**28.0** ± 1.52	**169.50** ± 4.63	**8.43** ± 0.24	**29.75** ± 2.42	**0.48** ± 0.02
Heavy metals	**45.80** ± 2.27 ***	**236.0** ± 16.98 **	**8.28** ± 0.25	**41.80** ± 1.98 **	**0.53** ± 0.02
Heavy metals +Propolis	**50.80** ± 2.92 ****	**229.0** ± 13.55 *	**8.04** ± 0.17	^^ **51.80** ± 0.86****	^ **0.45** ± 0.01

Values are presented as means ± standard error. Statistically significant differences ($p < 0.05$) compared to control group are indicated by *, while those compared to heavy metals group are indicated by ^. Statistical evaluation was performed using one-way ANOVA followed by Tukey's multiple comparisons test. * ^ *p < 0.05;* ** ^^ *p < 0.01;* *** ^^^ *p < 0.001;* **** ^^^^ *p < 0.0001*.

Table (3): Activities of investigated antioxidant enzymes U/g wet weight ± SE in liver and kidney of rats at experimental groups.

Groups	Enzymes / Organs	GPx	GR	SOD	CAT
Control	Liver	**196.94** ± 1.41	**61.13** ± 0.97	**34.50** ± 0.67	**227.50** ± 4.23
	Kidney	**55.74** ± 0.92	**28.89** ± 0.38	**13.82** ± 0.48	**356.30** ± 6.93
Heavy metals	Liver	**123.24** ± 1.72 ****	**27.23** ± 1.21 ****	**17.08** ± 0.70 ****	**98.75** ± 6.19 **
	Kidney	**26.90** ± 0.98 ***	**18.75** ± 0.51 ***	**5.68** ± 0.29 ***	**188.95** ± 9.73 ***
Heavy metals +Propolis	Liver	^^^ **175.85** ± 1.41 **	^^^^ **49.91** ± 0.90 ***	^^^^ **28.67** ± 0.77 ***	^^ **176.55** ± 5.35 *
	Kidney	^^ **45.38** ± 0.90*	^^ **25.23** ± 0.32*	^^^ **10.99** ± 0.36**	^^ **299.55** ± 5.71*

Observed Antioxidants enzymes are expressed on wet tissues as means ± standard error.. Statistically significant differences *(p < 0.05)* compared to control group are indicated by *, while those compared to heavy metals group are indicated by ^. Statistical evaluation was performed using one-way ANOVA followed by Tukey's multiple comparisons test. * ^ *p < 0.05;* ** ^^ *p < 0.01;* *** ^^^ *p < 0.001;* **** ^^^^ *p < 0.0001*.

Histopathological analysis:

Photomicrograph examination in the liver of control rats (Figure 1A) showed intact hepatocytes arranged in cord-like pattern (white arrow) separated by blood sinusoids (black arrow) and portal area (P). Treatment with the metal mixture at the heavy metal group **(**Figure 1B) resulted in; dilated blood sinusoids (black arrow), congestion and detachment endothelium of central vein (black arrow head), shrinkage and vacuolation of hepatocytes (white arrow) and necrosis of hepatocytes (white arrow head).

Furthermore, liver tissues of rats at propolis group (Figure 1C) showed mild dilation of blood sinusoids (black arrow), mild vacuolation of hepatocytes (white arrow) and intact endothelium of central vein (black arrow head). Additionally, photomicrograph illustration of kidney tissues at control group (Figure 2A) displayed intact renal corpuscle (white arrow) and renal tubules (black arrow). Meanwhile, metals administrated at the heavy metals group (Figure 2B) caused degenerated glomerulus with hyaline cast (white arrow) and degenerated renal tubules (black arrow). Unlike, renal tissues of rats at propolis group (Figure 2C) showed normal renal corpuscles (white arrow) and degenerated renal tubules (black arrow).

4. DISCUSSION:

Toxic metals and propolis:

In fact, deposition and accumulation of heavy metals among the soft tissues and internal organs of the body depend upon many factors like; the status of metals, their natures, introducing route, dose concentration and duration of exposure, as well as, their capability to react with cell proteins, consumer sensitivity and susceptibility.

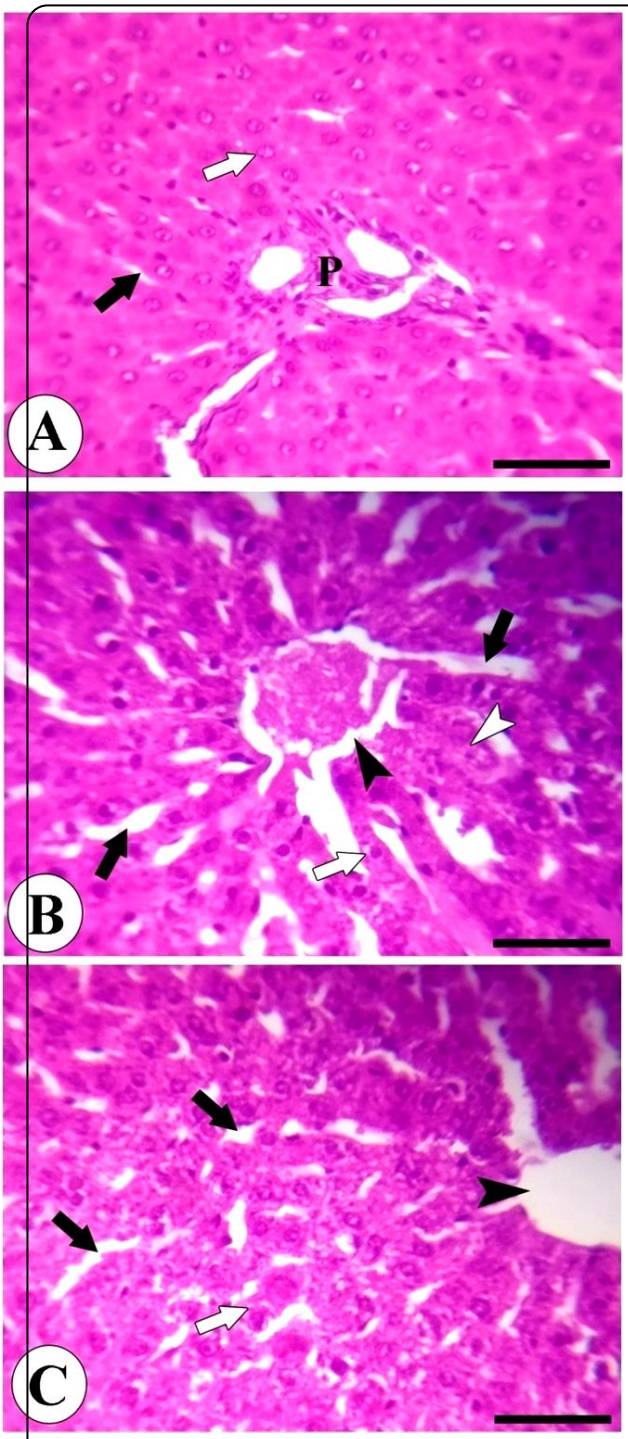

Figure 2. Effect of heavy metals mixture (Pb, Ni, Cd, Sb) on microstructures of rats' kidney after acute oral exposure. Panel (A): control group; panel (B): Heavy metals group; panel (C): Propolis group.

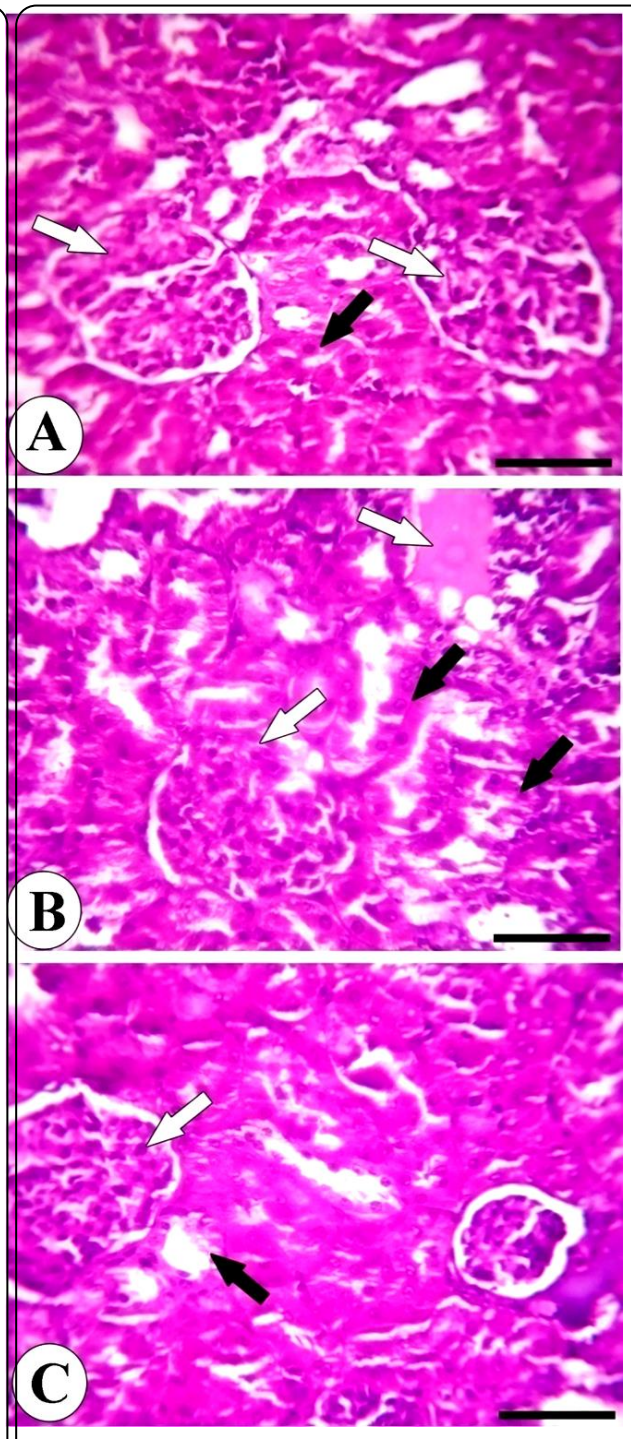

Figure 1. Effect of heavy metals mixture (Pb, Ni, Cd, Sb) on microstructures of rats' liver after acute oral exposure. Panel (A): control group; panel (B): Heavy metals group; panel (C): Propolis group.

Furthermore, livers and kidneys have the highest tendency to accumulate toxic metals after oral administration. In the most cases toxicity transport via blood and undergo intestinal absorption where they able to diffuse through red blood cells and cause severe health hazards. The present study has proved that the exposure to a mixture of metals in high levels (10x) for 75 days via oral administration may cause combined toxicity in male rats. Such toxicity biomarkers have attained significant decreasing after propolis treatment. Rats, exposed to metals mixture only, have exhibited high significant increase in the concentrations of these metals inside livers and kidney tissues ($p<0.0001$) in comparison to unexposed rats; the occurrence of early signs of oxidative stress in the current study has been observed after mixture treatment, this may be due to the capability of investigated metals to stimulate a disturbance in the oxidant and antioxidant balance inside the cells which results in whether excessive formation of free radicals such as singlet oxygen, hydroperoxides ($HO_2^{\cdot}$), and hydrogen peroxide (H_2O_2) or overproduction of ROS that cause increase in oxidative damages parameters, as well as decrease in normal antioxidant defenses, like GPx, CAT, SOD and GR, as clear signs for a cellular impairment, **[35 - 37]**. Besides, prolonged exposure to Cd may cause renal impairment through a decrease in the glomerular filtration rate and eventually may lead to renal failure rather than the ability of antimony to cause a loss in cell viability and other health effects involve the respiratory and cardiovascular systems likewise, nickel that is able to rise structural changes and functional disruptions in various tissues and organs of the body, **[38 - 40]**. Other possible reasons for combined toxicity and these observations might be due to the differences in the metals bioavailability and their competitive affinity to protein transporters following oral administration with possible synergistic involvements, [**41]**. On the other hand, the present data has detected that propolis treatment after oral exposure to metals mixture has afforded semi-normal recovering from many complications arose by metals effects where significant reduction in the concentrations of all investigated metals has been observed and confirmed statistically among liver tissues ($p < 0.001$) and kidney ($p < 0.01$) except cadmium which has been reduced without statistically significance. The depletion of lead, nickel, cadmium and antimony in the propolis treated group may be due to the antioxidant properties of propolis that are able to decrease the oxidative stress, inhibit progressive fluctuations convinced through metals mixture and repair alterations rising in the liver and kidney close to healthy measurements **[42].** Propolis can scavenge reactive oxygen and nitrogen species, prevent lipid peroxidation, upregulates biosynthesis of various cytoprotective and antioxidant proteins, and have an inhibitory effect on the inflammatory cytokines **[43 - 45].** Propolis has also shown a vital role in preventing damage to membranes or proteins as well as regulating their activity by interacting or regulating specific enzymes and influencing cellular structures in addition to, reactivating the defense characteristics of enzymatic antioxidants to protect hepatic, renal and various tissues from metals induced oxidative damage **[46].** Reversing the toxic effects of toxic metals is another beneficial property of propolis, as concluded by many authors **[47 - 49].**

Effect on biochemical attributes:

The present study has declared that heavy metals are able to cause alterations in the blood biochemical attributes. Administration of oral mixture metals to rats has resulted in significant differences in the investigated biochemical (decreased level of total protein; increased levels of urea nitrogen and creatinine, activities of ALAT and ASAT). There are no statistically changes in the level of total protein in the serum of rats from both experimental groups when compared to control however a slight depletion was observed in TP which is related to energy production during metals toxicity through metabolic utilization of the ketoacids to gluconeogenesis pathway for the synthesis of glucose, **[50 - 52].** Additionally, ***Seif et al.*** **[53]** revealed significant elevation in serum activities of TP levels in comparison to control rats. The present study has obtained significant elevation in urea level ($p < 0.01$) in the heavy metals group and higher significant level ($p < 0.0001$) after propolis treatment; this may be caused when heavy metals inhibit the activities of antioxidant enzymes and reach the soft tissues like kidney in the form of metal-metallothionein which is filtrated in the glomerulus, and subsequently reabsorbed in the proximal tubules. Then, it remains in the tubule cells and results in tubular damage, **[51, 54, 55].** Moreover, creatinine has shown a slight elevation after metals exposure, such elevation is a reflection of the degree of damage to glomerular filtration and is a sensitive biomarker for predicting kidney dysfunction, **[56 - 58].** However, createnine seems normal after propolis treatment**.** Furthermore, statistically significant elevation in the activity of ALAT enzyme has been observed in both experimental groups among liver ($p < 0.0001$) and kidney ($p < 0.001$) which may be attributed to subsequently releasing of mitochondrial enzymes into the blood as a result of tissues damage under toxic metals stress **[59, 60].** Similarly, high activity of ASAT in both groups has probably occurred under toxicity of heavy metals that cause different degrees of injuries in the liver leading to enzyme releasing

into the blood as biomarker of liver cell damage, **[37, 61].**

Histopathological effects:

Histological investigations have shown that the present concentrations of heavy metals mixture may cause relative damages to the tissues of rats at the heavy metal group. Only livers and kidneys of animals from the control area have almost shown no histopathological changes. Alterations in the liver tissue of rats from metals group can be attributed to the toxic effect of heavy metals mixture that affects liver functions causing loss of hepatocytes membrane integrity, liver enzyme elevations and reduction in the serum total protein in addition to, formation of highly reactive radicals and subsequent lipid peroxidation, which might cause cytotoxicity, **[62 - 64].** At the same time, relative damage reduction has been observed in the propolis group. Liver damages markers have been converted to; mild dilation of blood sinusoids, mild vacuolation of hepatocytes and intact endothelium of central vein, **[65, 66].** Additionally, renal damages have been distinguished in the contaminated heavy metals group appeared in degenerated glomerulus with hyaline cast and degenerated renal. These changes may be due to the accumulation of free radicals as the consequence of increased lipid peroxidation by free metal ions in the renal tissues which binds to metallothionein forming a complex released into the blood stream. This complex causes injury, mainly in the cortical region, reaching the proximal tubule and causing a gradual loss of the organ's function, **[67,68].** Nonetheless, these histopathological changes have decreased in the kidney of rats treated with propolis, consequently, mild degeneration and less necrosis in renal tubules have appeared with propolis administration as well as, normal renal corpuscles, **[**69**, 70].**

Antioxidants enzymes and propolis:

Dosing of heavy metals mixture may produce lower level of glutathione peroxidase (GPx) in liver ($p<0.0001$) and kidney ($p<0.001$) tissues at the heavy metals group in comparison with control group. This could probably due to the utilization of its defense mechanism against toxic metals within these organs as a result of ROS generation, **[71 - 73].** However, rats that have been exposed to toxic metals and treated with propolis have exhibited significant improve in the activities of GPx in livers ($p<0.001$) and kidneys ($p<0.01$) when compared with the heavy metals group; that issue clarifies the protective role of propolis in recovering the affected tissues of liver and kidney as healthy rats through reducing combined toxicity of investigated metals, **[74 - 76].** Additionally, after propolis treatment, both livers and kidneys of rats have exhibited statistical improve ($p<0.0001$ and $p<0.01$, respectively) in the activities of glutathione reductase (GR) which previously showed significant decrease among the livers ($p<0.0001$) and kidneys ($p<0.001$) of rats at the heavy metals group comparing to control, these fluctuations may be attributed to the ability of investigated metals to overcome the vital role of GR in order to interfere with the disulfide bond of glutathione enzyme and inhibit its activity, therefore, prevent the optimal balance and make cells more susceptible to oxidative damage, **[71, 77].** In line with this, activities of superoxide dismutase (SOD) enzyme has attained significant decrease in liver ($p<0.0001$) and kidney ($p<0.001$) tissues at the heavy metals group which might be interpreted to copper depletion which leads to decreased capability of cells to produce SOD, thus increasing their propensity to oxidative damage and disrupt SOD pivotal role on producing H_2O_2 in cells by a dismutation of superoxide radicals generated in the oxidative process, **[78, 79].** However, the antioxidant properties of propolis have led to statistically significant increase at the propolis group by $p<0.0001$ in livers and $p<0.001$ in kidneys comparing to the heavy metal group. Finally, catalase activity in the heavy metals group has almost been beneath the half of its activity in the control group within both liver ($p<0.01$) and kidney ($p<0.001$) tissues; this observation reflects how far the toxic metals affect catalase activity which has been relatively restored in the investigated organs from the propolis groups ($p<0.01$) by the action of antioxidant properties; the depletion of catalase is probably because of its ability to prevent toxic metal-induced consumption of O_2 inside cells, thus, capturing H_2O_2 before escaping out the cell and converting it to water and molecular oxygen. In this way, catalase can maintain the concentration of O_2 either for repeated rounds of chemical reduction or for direct interaction with the toxin, **[80, 81].**

Although several studies regarding propolis efficacy versus metals induced ROS toxicity have been conducted, there is an obvious lack of data on mechanisms underlying the propolis antioxidant properties against toxicity of some metals.

Conclusion:

The present results have shown a more profound toxicity of metal mixtures (Pb, Ni, Cd, Sb) via oral administration that induced toxic effects in the livers, and kidneys of adult *Wistar rats*. The main toxicity mechanism of combined metals is oxidative stress which is confirmed by a disturbed redox status and histopathological changes in the investigated tissues

of experimental rats, besides, clear biochemical alterations. The present study has also demonstrated that propolis is capable of reducing metals deposition inside livers and kidneys and improving biochemical alterations as well as histopathological alterations in addition to augment the activities of enzymatic antioxidants under investigation through many suggested mechanisms including lipid peroxidation inhibition, peroxidative prevention and neutralizing reactive species.

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Data availability:

The datasets from which the current study was created are available from the corresponding author on reasonable request.

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Abbreviations:

Abb.	Meaning
Pb	Lead
Ni	Nickel
Cd	Cadmium
Sb	Antimony
ROS	Reactive Oxygen Species
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
CAT	Catalase
SOD	Superoxide Dismutase
ASAT	Aspartate Transaminase
ALAT	Alanine Transaminase
TP	Total Protein
CAPE	Caffeic Acid Phenethyl Ester
MMP-9	Matrix Metalloproteinase-9
HNO_3	Nitric Acid
H_2O_2	Hydrogen peroxide
EEP	Ethanol Extracts of Propolis
HPLC	High Performance Liquid Chromatography
b.w	Body Weight
RPM	Round per minute
ANOVA	Analysis of Variance

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