UDC 575.
https://doi.org/10.2298/GENSR1901299A
Original scientific paper

EVALUATION OF PROTECTIVE EFFECTS OF PROPOLIS AGAINST ALUMINIUM SILICATE TOXICITY IN RATS

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Abd El-Aziz Y. M., A.H. Abu-Almaaty, N. A.Omar, A. M. Abdeen, M. M. Zakaria, (2019): *Evaluation of protective effects of propolis against aluminium silicate toxicity in rats.*- Genetika, Vol 51, No.1, 299-312.

Objective of the study was evaluation of the harmful effect induced by aluminum silicate in bone marrow chromosomes and liver tissues. Also, how could prevents this toxicity. 60 Adult male albino rats weighing 100-120 g were used for our experiments. The animals were divided into six equal groups: first normal healthy control group, second group was given 200 ml propolis/Kg day after day by stomach gavage, third group received low dose of aluminum silicate (5 mg/kg; intra peritoneal) twice a week, forth group injected with high dose of aluminum silicate (20 mg/kg; intra peritoneal) twice a week, fifth group received propolis and aluminum silicate with similar doses as that of second & third groups and sixth group received propolis and aluminum silicate with similar doses as that of second & forth groups. At the end of 8 weeks for each rat, bone marrow was aspirated and liver was removed for lab examinations. The results showed that rats exposed to aluminum silicate had severe chromosomal aberrations and changes in CYP gene expression. It caused significantly increased in the frequency of chromosomal aberrations such as polyploidy, hypoploidy, deletions, fragments, chromatid breaks, chromosome breaks, double minutes, ring, gap and translocation were also observed. Gene expression of CYPs was increased with exposure to aluminum silicate and was down regulated in

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treatment with propolis while Bcl-2 and BAX were decreased in exposure to aluminum silicate and up regulated by the treatment. Propolis has a curative effect against aluminum silicate toxicity owing to its antioxidant property.

Keywords: aluminum silicate, chromosome aberrations, gene expression, propolis.

INTRODUCTION

Aluminum is considered as the 3rd most abundant element approximately 8 % of total mineral compounds in the earth's crust found in combination with oxygen, silicon, nitrogen and other elements in the environment, stones, clays and jewelsand may have a significant toxicity possible for humans (VERSTRAETEN *et al.*, 2008). Silicon is the 2nd most common element which plays a central role in controlling the solubility of aluminosilicate minerals that constitute two thirds of the minerals in the earth's crust. These minerals are found commonly as feldspars in metamorphic and igneous rocks (MOLLER *et al.*, 2006). Aluminum is widely distributed in the soil and extensively used in daily life which causes easy exposure of human beings (KUMAR and GILL, 2009). The human body consumes aluminum through gastrointestinal and respiratory tracts. Aluminum is a constituent of cooking utilities such as ceramic pots and porcelain dishes that are using in various foods processing. It is also found in medicines such as antacids, phosphate binders, buffered aspirins, vaccines, antiperspirants and allergen injections (EXLEY, 1998). Aluminum silicate (AS) is considered as a dangerous compound because humans may consume it through the daily consumption via the diet, drinking water, ingestion with fruit juices or citric acid that causes a marked increase in both gastrointestinal absorption and urinary excretion of aluminum in healthy subjects (VENTURINI-SORIANO and BERTHON, 2001). Aluminum accumulates in all tissues of the humans including kidney, liver, heart, blood, bone and brain (AL-KAHTANI, 2010). The chronic exposure or long term aluminum silicate leads to the accumulation of metal abundance in the brain and liver as well as in other tissues and organs causing many metabolic and histological changes, membrane damage, altered gene expression and apoptosis. The liver is a critical organ because it contains most of the accumulated metals and where toxic effects can be expected (KURUTAŞ *et al.*, 2009).

Bees collect the propolis from different plant exudates and also known as bee glue in different temperate climatic zones. The worker bees gather the resin to seal any cracks and fissures in their hive for using in protection. They use it as an antiseptic in breeder cells, and they mix propolis with wax to distribute a fine varnish over every inch of the hives to prevent its contamination. Several empirical and clinical findings point to the fact that propolis may be effective against pathogenic microorganisms and environmental pollutes such as lead (AL-QAYIM *et al.*, 2013). The propolis has been used in folk medicine for antimicrobial, antiparasitic, antiviral, anti-oxidant, antitumor immune-stimulating and non-toxic natures (HENDI *et al.*, 2011). Thus, Greek and Roman physicians used it as mouth disinfectant and as an antiseptic and healing product in wound treatment, immune regulation, improvement of sleep, regulation of blood lipids and blood sugar. Propolis also improves gastrointestinal function, inhibit tumor, anti-fatigue, anti-aging and so many health benefits. Propolis has also been found to have powerful anti-inflammatory properties. Propolis can defense the damaging effects of aluminum (MAHMOUD and ELSOADAA, 2013). The chemical composition of propolis is more complicated containing more than 300 components such as flavonoids, phenolic acids and their esters, alcohols, ketones, amino acids, and inorganic compounds (SIMÕES *et al.*, 2004). Flavonoids are thought to be responsible for many of its biological and pharmacological activities (YOUSEF and

SALAMA, 2009). According to Orsolic, the chemical properties of propolis are not only beneficial to honey bees but have general pharmacological value to humans as a natural mixture. Propolis health food is useful component which regulate the physiological functions of the body plays a beneficial health effects (ORŠOLIĆ, 2010).

Numerous environmental and industrial pollutants are capable of causing cytogenetic aberration in experimental animals and humans. Aluminum is known to react with targets in the cell similar to those of known toxic metals like cadmium(MÜLLER and WILHELM, 1987). Hanafy represented that aluminum is considered as a genotoxic agent who evidenced by the increased incidence of chromosomal aberration particularly gaps and breaks in aluminum treated group rats. Aluminum caused lipid peroxidiation in the presence of iron (II) ion (Fe2+). Hydrogen peroxide (H2O2) greatly exacerbates the toxicity of aluminum**.** H_2O_2 induced chromosomal aberration. Propolis inhibited lipid peroxidation by aluminum in the absence of H_2O_2, but only decreased the process when H_2O_2 was present. There is an early demonstration that aluminum induces to chromosomal abnormalities, micronuclei and the exchange of sister-chromatids exchanges in human lymphocytes. KLINGELFUS *et al*., (2015) represented that Aluminum toxicity induced in the mononuclear leukocytes of people who used aluminum utensils on a daily basis to cook or store food leads to high degrees of DNA damage were related to oxidative stress in next generation (KLINGELFUS *et al.*, 2015).

MATERIALS AND METHODS

Propolis Extract

Propolis was procured from honey hives in Al-Arish city, Egypt. Propolis extract was prepared according to CUNHA *et al*. (2004). Thereafter, extract was administered orally to animal models via stomach gavage tube at a dose level of 200 ml/kg.

Experimental animals

The study was conducted after taking prior approvals from Helwan Animal Station (Ministry of Health, Egypt). Sixty adult male albino rats were divided into 6 equal groups. The selected rats were about 100 days old and weighting around 100-120 g maintained in plastic cages under controlled lighting conditions (12:12 light: dark cycle) relative humidity (50 ± 5 %) and temperature (24 ± 2˚C), fed with standard rat food that was given *ad libitum*. Aluminum silicate (AS) was purchased from Sigma-Aldrich (Cat. no. 520179, USA) and propolis was procured from honey hives in Al-Arish city, Egypt. The aluminum silicate doses were reconstituted and injected intraperitoneally while the propolis was administered by oral method via rat stomach gavage. The first group remained without any treatment and used as normal healthy group. The second group was given 200 ml propolis/kg day after day. The third group was received low dose of aluminum silicate (5 mg/kg) twice weekly. The forth group was injected with high dose of aluminum silicate (20 mg/kg) twice weekly. The fifth group was received propolis (200 ml/kg) in addition to exposure of low dose (5 mg/kg) aluminum silicate. Sixth group was received 200 ml/kg of propolis in addition to exposure of high dose aluminum silicate 20 mg/kg.

Detection of chromosomal abnormalities

Twenty-four hours after administration of last dose, animals were euthanized and bone marrow from femur was excised and air-dried metaphase preparations were done according to

the technique of Tijo and Whang (TJIO and WHANG, 1962; SINGH and SANKHLA, 2010). The slides were subsequently stained by 10% Giemsa dye (Stock solution: 3.8 g Giemsa, 250 ml glycerol, 250 ml methanol) and 100 metaphases per slides per animal were assessed. Hence, for each group a minimum of 500 metaphases were assessed for various chromosomal aberrations such as polyploidy, hypoploidy, deletions, fragments, chromatid breaks, chromosome breaks, double minutes, ring chromosome, gap and translocation. The slides were photographed under light microscope (Leica, Germany).

Real-Time PCR for CYPs, BAX and Bcl-2 genes:

Total RNA was extracted from liver of all rat groups of both controls and treated by SV total RNA isolation system (Promega Corporation, Madison, WI, USA) according to manufacturer's instruction. Then, the concentration of RNA was measured by spectrophotometer (Nanodrop 2000, Thermo Scientific, USA). RNA samples yielded only 2 distinctive bands in agarose gel electrophoresis and their relative absorbance 260/280 more than 2 and 260/230 more than 1.8 was intact and stored in liquid nitrogen tank. Thereafter, 2 µg of total RNA was converted to cDNA using RT^2First Strand Kit (QIAGEN Science, Maryland, USA). Real-time PCR for CYPs, BAX and Bcl-2 genes expression was done using commercial SYBR green master mix (Sensifast SYBR, Bioline, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control and for normalization.

Briefly, 2 µl of cDNA template, 10 pMol of each forward and reverse primer, 10 µl of 2X Master Mix and to 20 µl total volume by nuclease free water and then was introduced to thermal cycler instrument (Thermo Scientific, USA). The cycling parameters for the PCR amplification were achieved by initial denaturation at 95°C for 3 minutes followed by 40 cycles of 94°C for 15 seconds and annealing/extension step at 60°C for 1 min. For each gene test, the sample was carried out in triplicate. The primers design was performed on line at NCBI web site (Table 1). A mathematical model introduced by PFAFFL (2001) was used for the relative quantification of target genes. In this study, gene expression was expressed relative to that of normal healthy group.

Table 1. The primer sequences for Real-Time PCR assay

Gene	Sequences (5′-3′)	Gen bank Accession No.	PCR Products
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: CCAGGGCTGCCTTCTCTTGT R: CTGTGCCGTTGAACTTGCCG	NM_017008.4	123 bp
Cytochrome P450 (CYPs)	F: GGCACTCTGGACAAACACCT R: GGCCAGGAAGAGAAAGACCT	NM_012540.2	108 bp
Bcl-2 asseciated x protein (BAX)	F: GCAGACGGCAACTTCAACTG R: TGTCCAGCCCATGATGGTTC	NM_017059.2	120 bp
B-cell lymphoma 2 **(Bcl-2)**	F: GCAGACGGCAACTTCAACTG R: TGTCCAGCCCATGATGGTTC	NT_033907	120 bp

Statistical analysis

The nonparametric data were analyzed by program of SPSS 20 for Chi-Square independence test. The *P* value of < 0.05 was considered significant.

RESULTS

Cytogenetic study

In the present experiment, we studied the chromosome aberrations to assess the genotoxicity induced by aluminum silicate and to monitor the possible ameliorative effect of propolis. In the first, the normal healthy group as well as in the normal group receiving the propolis there were only normal chromosomes with 42 minor basal levels of aberrations (Fig.1). In the case of low dose of aluminum silicate, was observed chromosomal aberrations such as gap, deletion, double minutes, chromatid break, chromosomal hypoploidy and chromosomal polyploidy (Fig. 2). After addition of propolis, many of chromosomal aberrations such as rings, had lower frequency and most of the chromosomes were normal (Fig. 3). On the other hand, subsequently administered high dose of aluminum silicate induced more gaps, deletions, fragments, double minutes, translocations, rings and chromosomal breaks (Fig. 4), while the addition of propolis supplement caused significant decrease of deletions, double minutes, chromosome breaks, fragments, rings and gaps (Fig. 5). The satirical analyses of the total chromosomal aberrations is presented in Table 2 and the average of chromosomal aberrations for the experimental groups is showed in figure 6.

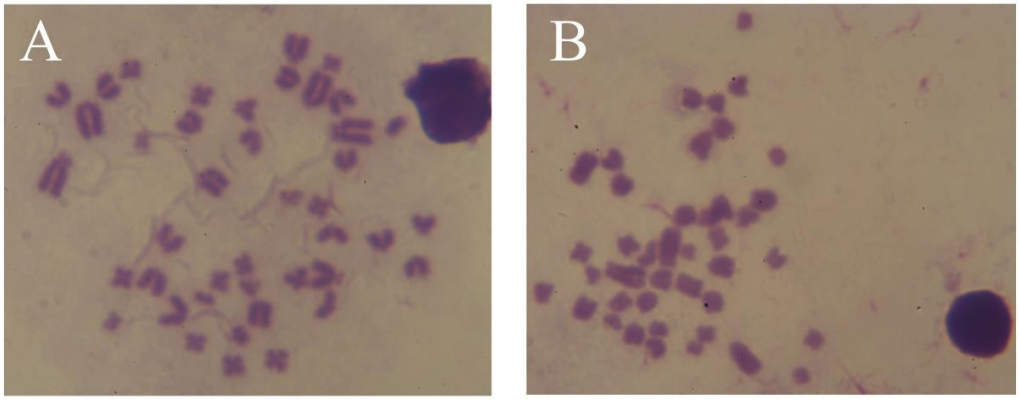

Figure 1. Metaphase spread of rat bone marrow cells for normal rat. A) Bone marrow cells of normal healthy control. B) Bone marrow cells of only oral propolis (200ml/kg).

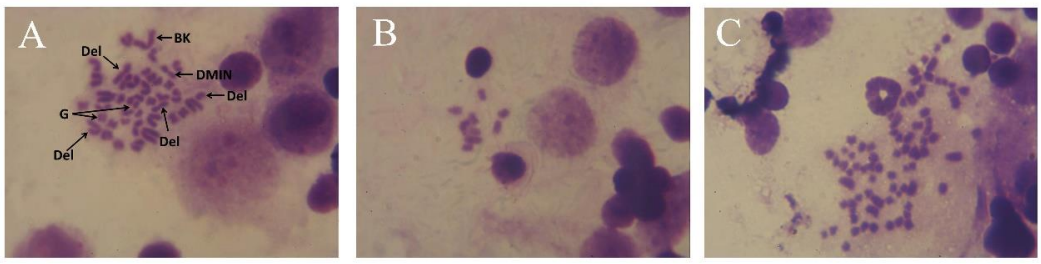

Figure 2. Metaphase spread of rat bone marrow cells for low dose of intraperitoneal aluminum silicate (5 mg/kg). A) Chromosomal aberrations: Gap (G), deletion (Del), double minutes (DMIN) & chromatid Break (BK). B) Chromosomal hypoploidy. C) Chromosomal polyploidy.

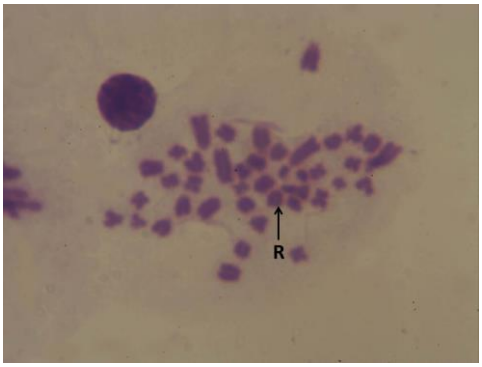

Figure 3. Metaphase spread of treated rat bone marrow cells with low dose of intraperitoneal aluminum silicate (5 mg/kg) in addition to oral propolis (200ml/kg). A) Mild of chromosomal aberrations: Ring (R) and many of normal chromosomes were observed.

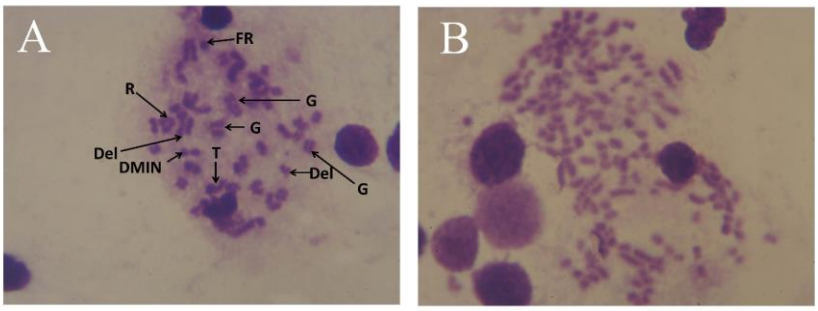

Figure 4. Metaphase spread of rat bone marrow cells for high dose of intraperitoneal aluminum silicate (20 mg/kg). A) Chromosomal aberrations: Gap (G), deletion (Del), fragments (FR), double minutes (DMIN), translocation (T), ring (R) & chromosome break (BK).

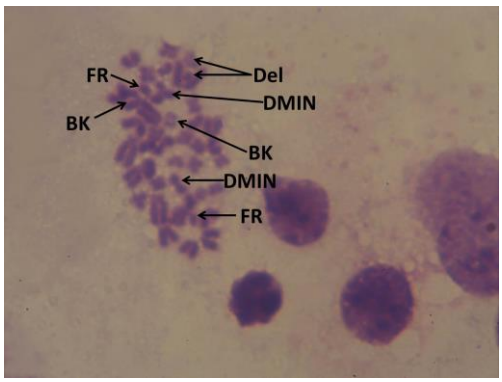

Figure 5. Metaphase spread of treated rat bone marrow cells with high dose of intraperitoneal aluminum silicate (20 mg/kg) in addition to oral propolis (200ml/kg). Deletion (Del), double minutes (DMIN), chromosome break (BK), fragments (FR), ring (R) and gap (G).

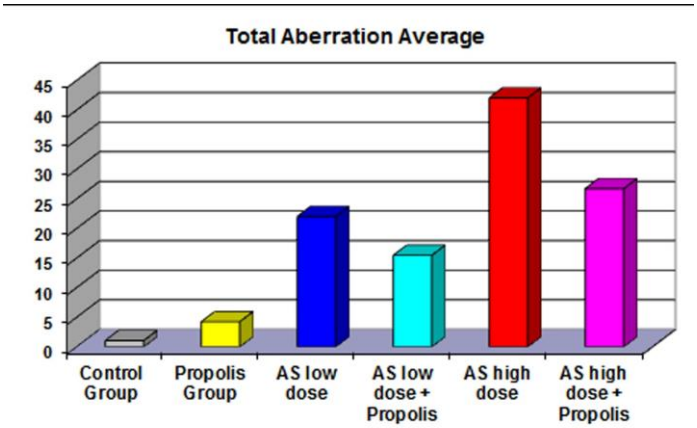

Figure 6. Total aberration average of bone marrow chromosomes for all rat groups.

Table 2. Chromosome aberrations in the different experimental groups

Experimental groups	Chromatid Gap	chromatid Break	Chromosomal Break	Deletion	Ring	Trans location	Double minutes	Fragment	Hypoploidy	Polyploidy
Control group	3.00	5.50	5.50	3.00	8.00	3.00	5.50	5.50	5.40	3.00
Propolis	10.10	5.50	5.50	8.00	3.00	8.20	5.50	5.50	5.60	8.00
AS low dose	19.10	16.60	15.10	20.30	21.10	23.00	18.00	18.00	20.10	19.10
AS low dose + Propolis	10.90	14.40	15.90	13.00	13.00	15.60	13.00	13.00	13.00	13.00
AS high dose	28.00	28.00	28.00	28.00	28.00	28.00	28.00	28.00	28.00	28.00
AS high dose + Propolis	21.90	23.00	23.00	20.70	19.90	15.20	23.00	23.00	20.90	21.90
Chi-Square	26.928	27.879	27.790	27.858	27.552	27.486	28.504	28.484	26.832	27.734
P value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

Metaphase score of cytogenetic aberrations of five readings (100 metaphase spreads for each rat). One, two or three stars is meaning non-significant, significant or highly significant, respectively. According to Chi-Square independence (Kruskal-Wallis H test).

Relative Gene Expression

The determination of CYPs, BAX and Bcl-2 expression was done relative to that of normal healthy control group and the mean of gene expression was calculated with standard deviation for all groups. The CYPs expression was elevated 1.47 fold in low dose group however by the addition of propolis, it was decreased to about 1.18 fold while at high dose of aluminum silicate the expression was up-regulated to 2.75 folds and by addition of propolis, CYPs gene was down-regulated to 1.87 fold of control (Fig. 7). Relative to normal control the expression of

BAX gene was down-regulated in both low and high dose by 0.10 and 0.08 fold while by administrated of propolis, the expression of gene was up-regulated to 0.20 and 0.24 fold (Fig. 8), respectively. Bcl-2 gene was also decreased in low and high dose of aluminum silicate by 0.53 and 0.36 fold whilst it was observed to up-regulated slightly by the dose of propolis to 0.54 and 0.51 fold, respectively (Fig. 9).

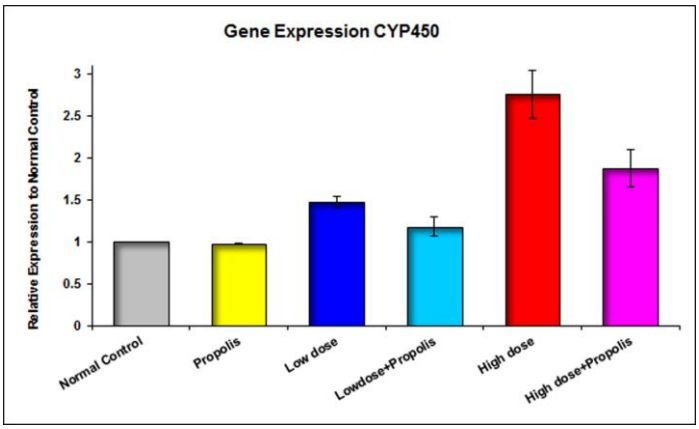

Figure 7. Relative Gene Expression of Cytochrome P450 for the mean of experimental groups with their standard deviation.

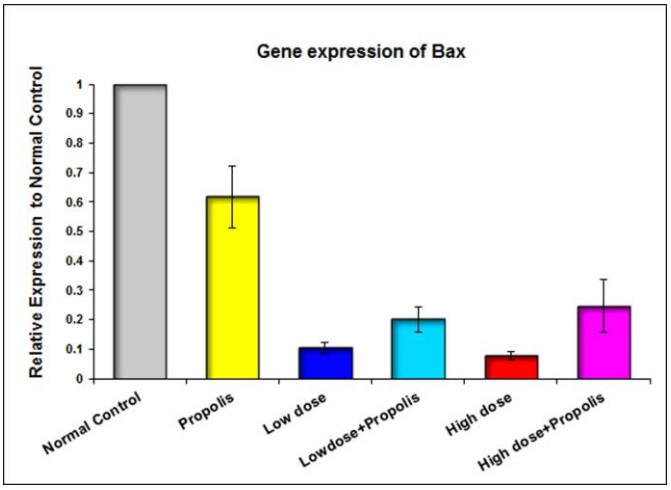

Figure 8. Relative Gene Expression of BAX for the mean of experimental groups with their standard deviation.

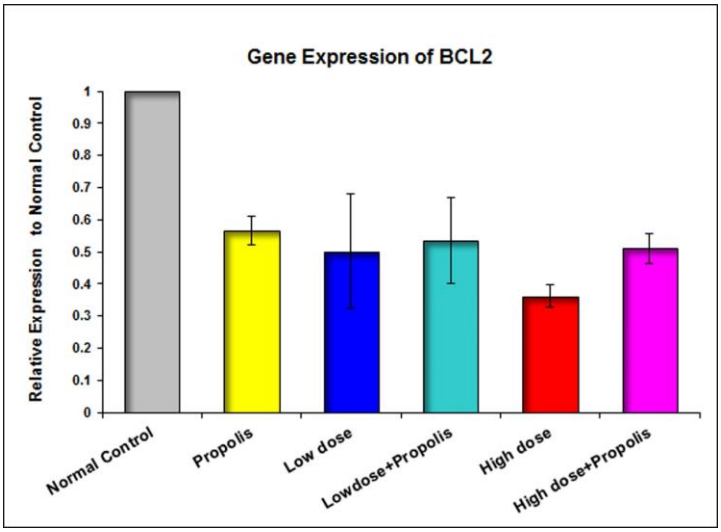

Figure 9. Relative Gene Expression of Bcl-2 for the mean of experimental groups with their standard deviation.

DISCUSSION

From beginning 20th century till now there is an increased use of chemical compounds that contains aluminum salts in industry and agriculture. Aluminum is found also in nature like air, water and soil and is considered the third most common element in Earth's crust. In other point of view, the wide range use of aluminum compounds that has led human to be exposed daily resulted a biohazard effects on human health (KUMAR and GILL, 2009; KUNO, 2009). Aluminum is known to have genotoxic profile capable of causing epigenetic disorders and DNA alterations as well as it is proved to be a metal with the capability to induce damage of DNA and disturbing balanced distribution of chromosomes during cell divisions (PAZ *et al.*, 2017). Moreover, aluminum silicate (AS) has a potential toxic effects in humans due to many daily uses in our life such as a constituent of cooking utensils such as ceramic pots and porcelain dishes as well as the exposure of its biohazard to workers in ceramic factories (MURPHY *et al.*, 1993). The low dose of AS was affected the bone marrow chromosomes by appearing of abnormalities up to three aberrations in each field (metaphase, 100X) with a total count 22.02% (per 100 metaphases). While at high dose of AS, it could induce more toxic effect up to 5 aberrations and the total aberrations was 42.06% (about 2 folds more). This finding is in accordance with HANAFY (2007) and PAZ *et al.*, (2017). However, using the supplement treatment (Propolis 200 mg/kg) dose at the same time with the AS compound there was a decrease of chromosomal aberrations. The number of total aberrations of low dose was decreased to average 15.46% for experimented rat group. On the other hand, the total aberrations of AS high dose in addition to propolis were decreased to about 26.73%. Therefore, propolis has anti-oxidant effect (AL-HARIRI, 2014; HERNÁNDEZ ZARATE *et al.*, 2018) in which aid to inhibit AS toxicity (SHAHNAWAZ and SANADHYA, 2017) in bone marrow chromosomes. Many authors agreed with our results whose proving the effect of this product in eliminating chromosomal aberrations in bone marrow, lymphocytes and hepatocytes of rat models (TÜRKEZ *et al.*, 2010; MONTORO *et al.*,

2012). For more details in different aberrations HANAFY (2007) was found the aluminum has affected the chromosomes by many aberrations such as inducing gap with a range of 4-5 per metaphase in each 100 metaphases scored and this is agreed with our works when we found the gap aberration was scored 2-5 per metaphase. Also, the break of chromatide in the same author was in a range of 6-7 and in our study the range was 3-7 per metaphase. The ploidy and deletion in HANAFY (2007) paper was 3-5 per metaphase however in our works the ploidy was 2-5 and deletion was 2-4 per metaphase. These finding of the genotoxic effect of aluminum is also in accordance with BALASUBRAMANYAM *et al.* (2009) and RAJESHWARI *et al*. (2015). In our experiment, the dosing of propolis for normal rats revealed no obvious changes for the bone marrow chromosomes while after exposure to AS, the administration of propolis minimized the chromosomal aberrations in both low and high dose of AS for different aberration types such as gap, total break of chromosome, deletion, ring, translocation, double minutes, fragment, hypoploidy and polyploidy. Therefore, propolis has ameliorative effects on chromosome aberrations of rat bone marrow. FEIBO XU *et al.* (2016) explain the importance of investigation of liver exposure to aluminum that promotes mitochondrial oxidative stress, which may be a contributing factor to mitochondrial energy metabolism disorder and liver dysfunction (XU *et al.*, 2017) and according to the anti-oxidant effect of propolis we investigated liver tissues through the apoptotic genes. The influence of AS and propolis in liver tissues was done by evaluating fold changes of mRNA of CYPs, BAX and Bcl-2. Cederbaum, 2017 described the decrease in the hepatic redox ratio, release of chemoattractants and cytokines, mitochondrial injury and bioenergetic impairment, induction of the cytochrome P450 enzyme CYP2E1, and other changes (CEDERBAUM, 2017). In our experiment the CYPs expression was elevated as it can generate reactive metabolites causing hepatocyte apoptosis by the exposure to AS, while in the presence of propolis CYPs expression was decreased indicating less apoptosis in liver cells. The pathway of CYPs during apoptosis was illustrated by many authors (HAOUZI *et al.*, 2000; LU *et al.*, 2012; DAS *et al.*, 2014). We found that, the effect of low dose AS was up regulated CYPs about 1.4 fold and down regulated to 1.1 fold by treatment of propolis. More confirmation by higher dose of AS elevated the CYPs expression to about 2.8 fold and decreased to 1.9 fold during the treatment. B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (BAX), anti-apoptotic proteins were down-regulated through low dose of AS and more inhibition by increasing a dose of AS. The finding is in accordance with other authors as RIZVI *et al*. (2014) was mentioned about the down-regulation of BAX and Bcl-2 expression in exposure to aluminum however in the present study, the treatment by using propolis showed a reduction of aluminum effect and leads to rise to gene expression in the hepatocytes. One point of view, the expression of BAX was affected with AS down regulated by low dose 0.1 fold and high dose by 0.07 fold less than expression in normal control however the Bcl-2 was less affected by AS induction 0.5 fold for low dose and 0.4 fold for high dose less than the normal one. These pathways need more explanations in next studies. In conclusion, The AS has a toxic effect in rat bone marrow and liver cells through chromosomal aberrations and inducing of apoptosis while, propolis has a prophylactic effect could minimize these toxicities.

CONCLUSIONS

Aluminum silicate has a toxic effect that leads to chromosomal aberrations in bone marrow and on other hand the propolis extract has a curative effect against this toxicity owing to its antioxidant property.

ACKNOWLEDGEMENTS

The study was done in Faculty of Science, Port-Said University and Urology & Nephrology Center, Mansoura University. This experiment was funded by us.

Received, February 22th, 2018
Accepted November 18th, 2018

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EVALUACIJA ZAŠTITNIH EFEKATA PROPOLISA PROTIV TOKSIČNOSTI ALUMINIJUM SILIKATA KOD PACOVA

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Izvod

Cilj istraživanja bio je procena štetnog dejstva indukovanog aluminijum silikatom u hromozomima koštane srži i tkivima jetre i kako se može sprečiti ova toksišnost. Za naše eksperimente korišćeni su odrasli mužjaci albino pacova težine 100-120 g. Životinje su podeljene u šest jednakih grupa: prva kontrolna grupa zdravih životinja, drugoj grupi je dato 200 ml propolis/kg svakog dana, treća grupa je primala nisku dozu aluminijum silikata (5 mg/kg intraperitonealno) dva puta nedeljno, četvrtoj grupi je data visoka doza aluminijum silikata (20 mg/kg; intraperitonealno) dva puta nedeljno, peta grupa je primila propilis i aluminijum silikat u sličnoj dozi kao druga i treća grupa, dok je šesta grupa primalapropolis i aluminijum silikat u dozama sličnim drugoj i trećoj grupi. Na kraju 8 nedelje iz svakog pacova, koštana srž je usisana i jetra je uklonjena zbog laboratorijskih ispitivanja. Rezultati su pokazali da su pacovi izloženi aluminijum silikatu imali ozbiljne hromozomske aberacije i promene u ekspresiji CIP gena. To je izazvalo značajno povećanje u učestalosti hromozomskih aberacija kao što su poliploidija, hipoploidija, delecije, fragmenti, prekidi hromatida, pauze u hromozomima, translokacije itd. Ekspresija gena CIPs je povećana sa izlaganjem aluminijum silikatu i smanjena je u tretmanu propolisom, dok su Bcl-2 i BAKS smanjeni pri izloženosti aluminijum silikatu, a ekspresija je povećana tretmanom. Propolis ima lekovito dejstvo na toksičnost aluminijum silikata zbog svog antioksidativnog svojstva.

Primljeno 22.II.2018.
Odobreno 18. XI. 2018.