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Betulinic acid and rotundic acid alleviates genotoxicity induced by mercuric chloride on blood lymphocytes of albino wistar rats

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Abstract
Mercury is an uncommon element present in the universe, known to be greatly dangerous in comparison to other heavy metals. Hg is highly toxic to the organisms, causing severe damages to most of the cells, tissues and organs. Thus, the present investigation was framed to find out the ameliorative efficacy of betulinic acid and rotundic acid on genotoxicity induced by mercuric chloride on blood lymphocytes of adult albino male rats. $HgCl_2$ was given orally for 1.29 mg/kg of body weight of Albino wistar rats for 7 days and betulinic acid and rotundic acid (5mg/kg body weight) was dosed for another 7 days on intoxicated rats. The genotoxicity induced by $HgCl_2$ was analyzed through comet assay and RT-PCR expression of 8-oxoguanine on blood lymphocytes of rats. The results revealed that betulinic acid and rotundic acid treatment significantly improved the defense system by enhancing their antioxidant properties and attenuate the DNA damage prompted by $HgCl_2$.

Keywords: mercury chloride, DNA damage, comet assay, 8-oxoguanine, betulinic acid and rotundic acid

Introduction
Ecological and worldwide national health care efforts are recently focusing on the heavy metal contaminations in the environment. Expanding utilization of heavy metals in numerous industries, including agriculture and technology, has dramatically increased human exposure (Abdel–Aty *et al*., 2013) [1]. Exposure of living organisms to mercury goes back several decades still now for various purposes (Mutlu *et al*., 2012) [29]. Mercury is an uncommon element present in the universe, known to be highly toxic in comparison to other heavy metals (Dash and Das, 2012) [10]. Practically all forms of Hg are highly toxic to the organism, causing severe damage in most of the organs and tissues (Clarkson and Magos, 2006) [7]. It is a sensitive metal that has a higher affinity towards the macromolecules and binds with DNA, prompting alterations in the DNA structure (Li *et al*., 2006) [23]. Mercury induced nuclear and genetic alterations, such as depleting DNA synthesis and damages DNA, additionally it was also reported that treatment of mercury induces DNA single-strand breaks even at low concentrations in mammalian cells, moreover changes in RNA and protein synthesis and results in apoptosis (Rhyaf, 2016) [38]. Mercury performs as a genotoxin through altering gene expression that affects cell survival and apoptosis (Shenker *et al*., 2002) [42]. The toxic effects of mercury can be prevented to some extent either by chelating or enhancing antioxidant defense mechanisms (Pillai and Gupta, 2005) [34]. Antioxidants provide protection against deleterious effect of free radical attack in the body (Agarwal *et al*., 2010) [2]. The oxidative damage of DNA, proteins and lipids through reactive oxygen species (ROS) has been the recent focus of research studies extensively, since it has been implicated in many common diseases, such as diabetes, Alzheimer's, cardiovascular, Parkinson's, cancer, acute renal failure, Down syndrome and autism (Hepel *et al*., 2012) [21].

Guanine has a lower redox potential than the other three bases, which makes it especially susceptible to oxidation (Neeley and Essigmann, 2006) [31]. A radical attack at the 8'-carbon of guanine gives the oxidation product 8-oxoguanine, which is the most commonly measured oxidative DNA base lesion and an established biomarker for oxidative stress (Pouget *et al*., 2000) [36]. 8-oxoguanine pairs with cytosine in the regular anti conformation, yet can in its syn conformation form a stable base pair with adenine (David *et al*., 2007) [11]. The comet assay (single-cell gel electrophoresis) has become one of the methods of preference for the assessment and estimation of DNA damage (Møller, 2018) [27], it is also quite simple, quick, cost effective, accurate and reliable (Pu *et al*., 2015) [37]. Therefore, comet assay and 8-oxoguanine expression through RT-PCR analysis were performed to assess the DNA damages induced by $HgCl_2$.

Natural products have traditionally formed the backbone of modern drug discovery programs. Betulinic acid (BA), a natural pentacyclic triterpene present in all the plant kingdoms. Previous studies evident that, BA possess numerous biological properties like anti-tumor, anti-inflammatory, anti-HIV, anti-bacterial, anti-fibrotic, anti-malarial, anti-angiogenic, anti-ulcer and anti-oxidant properties (Wan *et al*., 2012) [50]. BA influences protective role towards cell apoptosis by diminishing oxidative stress via regulating the signaling pathway mediated by mitochondria was revealed by previous investigation (Yi *et al*., 2016) [56]. Rotundic acid, a plant-derived pentacyclic triterpene, is broadly present in edible and medicinal plants (Yang *et al*., 2018a) [54]. RA also has a various and extensive pharmacological properties, including anticancer, anti-inflammatory, antidiabetic, antibacterial, antimycobacterial, lipid-lowering properties, etc. (Shang *et al*., 2021) [41].

There are no records or data related with the ameliorative effect of betulinic acid and rotundic acid against

genotoxicity (DNA damage) induced by $HgCl_2$. Therefore, the present investigation targeted on the protective potential of betulinic acid and rotundic acid against genotoxicity induced by $HgCl_2$ on the blood lymphocytes of male albino rats.

Materials and methods

Chemicals utilized

Mercuric chloride ($HgCl_2$) and further more vital reagents for investigative evaluation were acquired from Hi-Media laboratories Ltd, Mumbai, India. Betulinic acid and rotundic acid were purchased from Sigma Aldrich laboratories Pvt. Ltd, Bangalore, India.

Adaptations of animals

Healthful adult male albino rats, *Rattus norvegicus* of body weight between180–200 g were acquired from the Central Animal House, Department of Experimental Medicine, Raja Muthiah Medical College and Hospital, Annamalai University, and kept up in a cool air conditioning room (25 ± 3◦C) with a 12 hrs lighted and 12 hrs dull cycle. Feeds, water and *ad libitum* were supplied to all the rats. The experimental protocols were examined and accepted by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (IAEC, Proposal Number: AU-IAEC/1228/1/19), Annamalai University, Annamalai nagar.

Experimental design

Total of 36 wistar rats were acclimatized in the animal cages for 7 days. They were rifled and partitioned into six gatherings, each comprise of six rodents. The toxic dose of mercuric chloride has been resolved (sub-lethal dosage of $HgCl_2$ 1.29 mg/kg bodyweight of the animal) from our past examination conducted in our laboratory. Furthermore it has adequate to evoke gentle or mediated oxidative stress in rodents 36.

- Group I: Untreated control- just coursed (0.9% NaCl) was given to the animals and watched for 7 days.
- Group II: Mercuric chloride treatment- the animals were managed with 1.29 mg of $HgCl_2$/kg body weight of the animal in 0.9% NaCl intraperitoneally for 7 days.
- Group III: Mercuric chloride followed BA treatment- the animals were managed with betulinic acid (5 mg/kg body weight of the animal) after the inebriation of mercuric chloride for 7 days.
- Group IV: Mercuric chloride followed RA treatment- the animals were managed with rotundic acid (5 mg/kg body weight of the animal) after the inebriation of mercuric chloride for 7 days.
- Group V: BA treatment alone- the animals were given betulinic acid (5mg/kg body weight of the animal) alone for 7 days.
- Group VI: RA treatment alone- the animals were given rotundic acid (5mg/kg body weight of the animal) alone for 7 days.

Towards the end of the experimental program, blood samples were collected in clean dry test tubes through the sinoauricular punch of the animals. From the blood samples the lymphocytes were freshly isolated through density gradient method and used for the following analyses.

Comet Assay

The severity of DNA damage was estimated by alkaline single cell gel electrophoresis (Comet assay) according to the method of Singh (1988) [43] with slight modifications. Protracted slides were prepared by pouring 3.0-5.0 ml of 1% normal agarose over clean glass slide. It was allowed to dry at room temperature and placed in hot-air oven at 70-80ºC for 30 min. The freshly prepared control and experimental lymphocytes were mixed with the second layer of 200 µl of 0.5% low meting agarose (LMA). Cover slip was placed immediately and the slides were removed and slides were placed in the chilled lysing solution for 1 h at 4ºC. After lysis slides were placed in a horizontal electrophoresis tank. The unit was filled with electrophoresis solution for 20 min to allow DNA unwinding electrophoresis was conducted in a cold condition for 20 min at 25v and 300mA after electrophoresis the slides were placed horizontally and neutralized with tris-HCL buffer. Finally, 50 µ of propidiuam iodide was added to each slide and analysed using a fluorescence microscope to prevent additional DNA damage all steps were conducted under dimmed light or in the dark. Fifty comet images were randomly selected from each sample and were examined at 200x magnification in a florescence microscope connected to a personal computer based image analysis system. The quantification of the DNA stand breaks of the stored images was done by using the caps software by which % DNA in tail, tail length, tail moment and olive moment could be obtained directly. The relative amount of DNA appearing in the tail of the comet (percent tail DNA), tail length, tail moment and olive tail movement were linearly related to DNA breaks frequency.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using guanidiumthiocyanate-chloroform-phenol according to the method of Chomczynski and Sacchi, (1987) [6]. The freshly collected blood lymphocytes sample of control and treated groups were treated with RNA isolation buffer (1:1 ratio). The sample was transferred to a 15 ml polypropylene tube and added in order: 2.5 ml of 2.5 M sodium acetate (pH 4.6), 0.5 ml of saturated phenol (80%) and 2.5 ml $CHCl_3$: Isoamyl alcohol (24:1). Following the addition of each reagent, the contents were mixed thoroughly by inversion. Cool the sample on ice for 15 min, the samples were centrifuged at 10,000 rpm for 15 min at 4˚C. To the aqueous phase equal volume of ice-cold isopropanol was added and kept at -20˚C for 1h. The RNA was precipitated at 12,000 rpm for 15 min at 4˚C, discard the supernatant and the pellet washed with 80% ethanol. The resulting pellet was dried briefly in vacuum and dissolved in minimal volume of sterile DEPC treated MQ water. The amount of RNA was quantified by using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

RT-PCR for the mRNA expression levels of 8-Oxoguanine (8-oxoG) in the blood lymphocytes of control and experimental animal group were performed using RT-PCR detection kit (Sigma Aldrich, USA) in a Realplex Mastercycler (Eppendrof, Indianapolis, CN). The Primer sequences for 8-oxoG: Forward: 5'-GATTGGACAGTGCCGTAA-3'; Reverse: 5'-GGAAGTGGGAGTCTACAG-3' and GAPDH: Forward: 5'-ACACCCACTCCTCCACCTTT-3' Reverse: 5'-TAGCCAAATTCGTTGTCAACC-3' a housekeeping gene expressions were used for normalization. To the analysis of mRNA expression in the blood lymphocytes of control and experimental groups of rat were determined by using RT-PCR, as described previously (Ponchel *et al*., 2003) [35].

Experiments were run in triplicate to conform amplification integrity. Manufacturer-synthesized primer pairs were used to measure the mRNA expression. For cDNA synthesis, PCR cyclic condition used were 25˚C for 10 min; 42˚C for 50 min; 75˚C for 15 min. The cyclic condition used for cDNA amplification was 95˚C for 15 sec; 55˚C for 15 sec; and 68˚C for 20 sec. The expression levels of genes were normalized to GAPDH mRNA expression level. Images were acquired by using Kodak GL 100 imaging system. Equal quantities of DNA sample were mixed with an appropriate volume of 1X loading buffer electrophoresed through a 2% agarose gel in 0.5 % TBE buffer. Electrophoresis was carried out at 5 V/Cm. DNA in the gels was visualized by adding 1 mg/ml of EtBr. Bands obtained were analyzed using Kodak GL 100 imaging system. Expression obtained from the samples of control animals were counted as 100%. Relative percentages of expression from the samples of experimental groups were calculated. The expression band intensity has been scanned by the densitometer.

Statistical Analysis

Data values are expressed as mean ± S.D. for six animals in every group. T-test analysis was used to analyze various data acquired from assorted biochemical parameters, and the group means were correlated by Duncan's multiple range test (DMRT) (Duncan, 1957) [12]. Obtained values were evaluated statistically significant when $p < 0.05$, and the values sharing a general superscript did not significantly differ.

Results

Image analysis of Comet assay

In the present experimental work, exactly hundred comets were scored (50/slides and 2 slides/rat) through the Computer automated image analysis systems for each treated and control group. Based on the type of evaluation results of comets by image analysis are shown in Table 1. The changes of comet tail DNA%, tail length and tail moment with applied $HgCl_2$, $HgCl_2$ + Betulinic acid, $HgCl_2$+ Rotundic acid, Betulinic acid alone, Rotundic acid alone and untreated control groups are shown in the Figure 1. The evaluation of lymphocytes nuclei with the comet assay demonstrated that mercuric chloride treatment caused significantly higher DNA damage in comparison with untreated control. After the scheduled treatment, betulinic acid and rotundic acid significantly decreased the $HgCl_2$-induced DNA damage when compared to the $HgCl_2$ intoxicated group (Figure 1, 2 and Table 1).

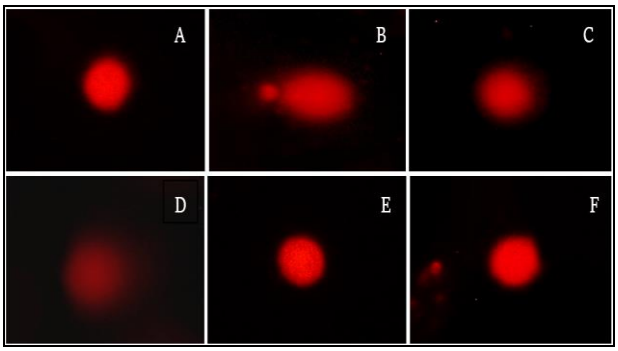

Fig 1: Comet assay images showing the ameliorative potential of betulinic acid and rotundic acid on lymphocyte DNA damages in $HgCl_2$ intoxicated rats. A – Control shows no DNA damage; B – $HgCl_2$ shows extensive DNA damage; C – $HgCl_2$ + Betulinic acid shows moderate DNA damage; D – $HgCl_2$ + Rotundic acid shows moderate DNA damage; E – Betulinic acid shows mild change; F – Rotundic acid shows mild change.

Table 1: Illustrates the protective effect of betulinic acid and rotundic acid on mercuric chloride intoxicated rats DNA damage

Groups	Head	Tail	Tail Movement	Olive Tail Movement
Control	99.01±0.11^a	0.15±0.04^a	0.08±0.03^a	0.05±0.12^a
$HgCl_2$ Treatment	31.21±0.04^b	34.72±0.02^b	69.38±0.04^b	63.23±0.08^b
$HgCl_2$ + Betulinic acid	96.36±0.04^c	5.47±0.02^c	2.28±0.01^c	1.12±0.08^c
$HgCl_2$ + Rotundic acid	93.19±0.02^c	12.65±0.06^d	17.30±0.04^d	8.21±0.12^d
Betulinic acid	98.87±0.12^a	1.1±0.04^e	0.79±0.03^a	0.16±0.11^a
Rotundic acid	98.77±0.02^a	1.23±0.06^e	0.83±0.01^a	0.46±0.10^a

Values are expressed as mean ± SD; values are taken as a mean of six individual experiments values not sharing a common superscript letter or differ significantly (DMRT)

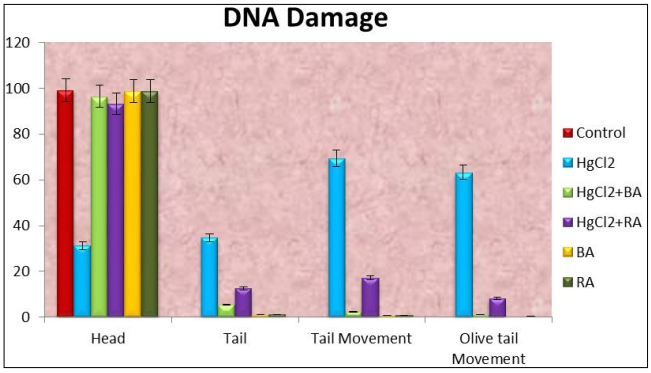

Fig 2: Illustrates the DNA damage were analyzed by images software (CAPS)

RT-PCR expression of 8-oxoguanine (8-oxoG)

Figure 3 and 4 shows the expression of 8-oxoguanine (8-oxoG) in the blood lymphocytes of control and mercuric chloride intoxicated experimental rats. The lymphocytes sample of rats intoxicated with $HgCl_2$ showed increased level of 8-oxoG expression when compared to control animals. Post-treatment with betulinic acid and rotundic acid against mercuric chloride intoxicated rat blood lymphocytes exhibited the down regulation of 8-oxoG expression when compared to $HgCl_2$ intoxicated rats. Betulinic acid and rotundic acid alone treated blood lymphocytes of rats were showed no changes on 8-oxoG expression when compared to control animal. Densitometry analysis of RT-PCR is given in Figure 3 and 4.

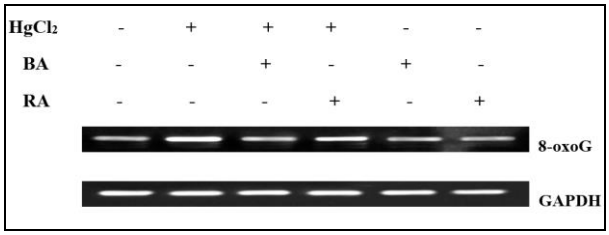

Fig 3: Illustrates the Influence of betulinic acid and rotundic acid on mercuric chloride intoxicated rat blood lymphocytes 8-oxoG expression.

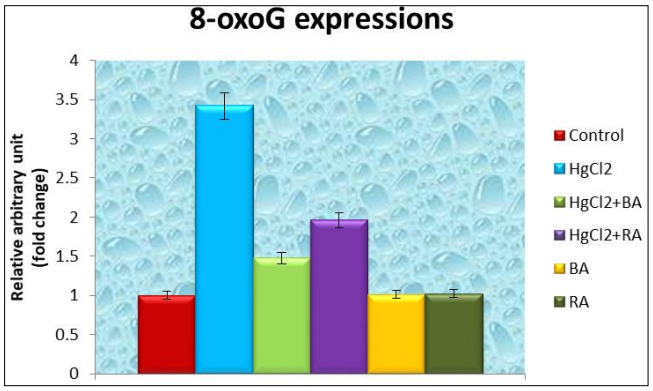

Fig 4: Shows the statistical representation of 8-oxoG expressions Band intensity scanned by densitometer

Discussion

Mercury (Hg) is recorded as one of the significant chemicals of incredible public health concern (World Health Organization, 2005) [51]. Once absorbed, Hg is quickly transported in red blood cells, because of its greater membrane permeability, and distributed widely throughout the body, especially in the liver, central nervous system (CNS) and kidneys (Nabi, 2014) [30]. Curiously, few investigations hypothesize that oxidative stress can create this adaptive response (Ristow and Schmeisser, 2014; Stokes-Riner *et al*., 2011) [39, 44]. Li *et al*., (2006) [23] illustrated that Hg species can directly interact with DNA molecules via covalent interaction of endocyclic and exocyclic N sites of DNA bases. Therefore, to better understand the genotoxic effects of inorganic Hg in rats, the comet assay could serve as the indicator of DNA damage caused by $HgCl_2$. There are only some studies on the genotoxicity of mercury and its derivatives, and the general assessment of results is very perplexing and hard to interpret (Bhowmik and Patra, 2015) [5]. A number of previous *in vitro* and *in vivo* studies described that the compounds which improve the redox status through induction of antioxidant enzymes are protective against DNA damage caused by mercury and other metals (Barcelos *et al*., 2011) [4]. Therefore, betulinic acid and rotundic acid with greater antioxidant properties were used to protect from the DNA damage induced by $HgCl_2$.

In the present investigation, intensity of DNA damage was high with reduced head region but increased tail, tail movement and olive tail movement in the lymphocytes of rat intoxicated with $HgCl_2$. The results of the comet assay obtained after sub-lethal treatments with $HgCl_2$ was seen to be increase in the dose of $HgCl_2$ resulted in the increase of damaged DNA showing a clear dose–response relationship. Similarly, appearance of Hg induced comet was qualitatively different from those of control cells (Bhowmik and Patra, 2015) [5]. Mercury exposure has been correlated with oxidative stress on some *in vivo* studies (Crespo-Lopez *et al*., 2007; Franco *et al*., 2007) [9, 15], in which mercury induces the formation of reactive oxygen species (ROS) and it modify the antioxidant defense system of cells by inhibiting their sulfhydryl groups (-SH) (Mori *et al*., 2007) [27]. The ROS attacks DNA by altering the hydroxyl radical (.OH), superoxide anion (O2-.), and hydrogen peroxide, frequently prompt oxidative DNA damage (Grotto *et al*., 2009) [17]. Therefore, the administration of betulinic acid and rotundic acid therapy followed by mercury chloride reduced the DNA damage in the lymphocytes. The present experimental work proves that BA and RA treated cells significantly exhibited the reduction in the percentage of DNA tail length and tail movement compared to the mercuric chloride-treated group, and thus DNA protective effect is evident. Moreover, these dietary natural compounds protect DNA against the deleterious effects of physical agents, ionizing radiation, and toxins and chemotherapeutic agents. In fact, DNA damage is one of the important, as well as malicious, outcomes of exposure to these agents. Since one of the significant reason for DNA damage is the increased ROS levels, the protective potency of polyphenols are presumably related to their antioxidant potential (Majidiniaa *et al*., 2019) [25].

Mercury and its compound are appears to have a mutagenic potential (Mergler *et al*., 2007) [26]. Thimerosal induces important sister chromatid exchanges, showing a genotoxic and cytotoxic impact of thimerosal in a cultured human peripheral blood lymphocytes (Eke and Celik, 2008) [13]. Previously it was reported that, Hg can strive toxicity through alteration of gene expression, as well as modifying the profile of gene expression that happens as a cellular reaction to Hg exposure. These chemical interactions between Hg and nucleic acids have been reported in detail (Onyido *et al*., 2004) [33]. Accordingly, it was hypothesized that DNA and/or RNA could be targets of Hg through which its toxicity is intervened (Ueda *et al*., 2014) [48]. Ali *et al*., (2018) [3] reported that mercury chloride possess genotoxic impacts on the cells. The damaged DNA is a key initiator for tumor growth and development. The stability between induction of lesion and repair determines DNA oxidation levels. Proliferated oxidative stress or dropped repair prompts elevated DNA lesions and mutation accumulation that promotes cancer. Furthermore, mercury performs as a mitotic spindle inhibitor through binding with the SH groups in eukaryotes. ROS developed by mercury increases DNA damage, carcinogenicity and tumorigenicity (Gregory and Pound, 2011) [16].

8-oxoguanine (8-oxo G) can act as a biomarker to assess the risk of oxidative stress in the present experimental study. The results shows an elevated expression of 8-oxoG in the lymphocytes of rat intoxicated with $HgCl_2$. Excessive mercury concentrations might produce translational inhibition besides to produce transcriptional inhibition. This variance could be clarified by dissimilarity in structure; generally mRNA is single-stranded and thus possesses fewer positions for Hg-mediated cross-bridging than double-stranded DNA. Furthermore, ribosomes on the mRNA have the ability to resolving Hg-mediated secondary structures that prevent protein translation (Ueda *et al*., 2014) [48]. Mercury exposure and its contamination are considered as effective promoters of health problems; ranging from physiological disorder to the mental issues. For example, mercury exposure causes central nervous system defects and erethism as well as arrhythmias, respiratory failure, cardiomyopathy and kidney damages (Harari *et al*., 2012) [19]. Furthermore, Hg could act as an immunostimulant and suppressant factor, giving pathologic sequelae such as hypergammaglobulinemia, lymphoproliferation and total systemic hyper- and/or hypo-reactivities (Clifton, 2007) [8]. These genes were verified by the increased mRNA expression levels shown by real time polymerase chain reaction (RT-PCR) (Rudgalvyte *et al*., 2017) [40]. Similar to our findings, in the study of Xu *et al*., (2017) [52] the levels of 8-hydroxydeoxyguanosine (8-OHdG), one among the

biomarker for DNA damage was found to be higher in the blood of Hg exposed children than that of control groups. Experimental investigation of Turkez *et al*., (2012) [47], determined that MMC (mitomycin C) triggers the development of oxidative stress and increases the level of 8-OH-dG in the human blood lymphocytes.

Henceforth, betulinic acid and rotundic acid reduces the impact caused by mercury chloride on the DNA of rat's lymphocytes. Betulinic acid has been reported to possess protective shield for normal cells, like peripheral blood lymphoblasts, human peripheral blood mononuclear cells, human astrocytes, leukocyte, liver stellate cells, dermal fibroblasts and melanocytes (Ekşioğlu-Demiralp *et al*., 2010; Viji *et al*., 2011; Wan *et al*., 2012) [14, 49, 50]. It was perceived that BA can scavenge ROS and protect hepatocytes from the oxidative damage induced by ethanol, carbon tetrachloride, cadmium and acetaminophen on *in vivo* and *in vitro* studies (Szuster-Ciesielska *et al*., 2005, 2011; Yi *et al*., 2014; Yoon *et al*., 2010; Zheng *et al*., 2011) [45, 46, 55, 58, 59]. Therefore, BA was believed to play a major role as a dietary immunomodulator and radical scavenger for the prevention of oxidative damage in living systems (Yi *et al*., 2015) [57]. Furthermore, rotundic acid have been reported for the various biological activities such as, anti-tumor (Xu, 2009) [53], blood pressure lowering (Liu, 2010) [24], and in the prevention and treatment of cardiovascular disease (He *et al*., 2013) [20]. Thus, all together BA and RA treatment in the present study enhances the DNA repair mechanism by improving the antioxidant status and reduces the oxidative DNA damage (8-oxoguanine) in blood lymphocytes of rats intoxicated with mercuric chloride.

Conclusion

The present study suggests that betulinic acid and rotundic acid has the potentiality to ameliorate DNA damage induced by mercuric chloride. The role of the natural antioxidants in the betulinic acid and rotundic acid may influence the protective properties by possibly up-regulating the level of the antioxidant defense system and abolishing oxidative DNA damage through the donation of electrons to reactive metabolites and rendering them inactive to prevent the interaction to the DNA. The experiment on the protective role of phenolic compounds as an excellent radical scavenger to reduce the DNA damage in the blood lymphocyte is also consistent with this result. As a whole, our results propose that betulinic acid and rotundic acid might be promising compounds for the treatment of genotoxicity induced by mercury chloride.

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Conflicts of interest

The authors declare that there are no conflicts of interest concerning this article.

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