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ORIGINAL ARTICLE

In vitro cytogenotoxic evaluation of sertraline

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ABSTRACT

Sertraline (SRT) is an antidepressant agent used as a neuronal selective serotonin-reuptake inhibitor (SSRI). SRT blocks serotonin reuptake and increases serotonin stimulation of somatodendritic serotonin 1A receptor (5-HT1AR) and terminal autoreceptors in the brain. In the present study, the genotoxic potential of SRT was evaluated using cytokinesis-block micronucleus (CBMN) cytome assay in peripheral blood lymphocytes of healthy human subjects. DNA cleavage-protective effects of SRT were analyzed on plasmid pBR322. In addition, biochemical parameters of total oxidant status (TOS) and total antioxidant status (TAS) in blood plasma were measured to quantitate oxidative stress. Human peripheral blood lymphocytes were exposed to four different concentrations (1.25, 2.5, 3.75 and 5 µg/mL) of SRT for 24- or 48-h treatment periods. In this study, SRT was not found to induce MN formation either in 24- or 48-h treatment periods. In contrast, SRT concentration-dependently decreased the percentage of MN and MNBN (r=–0.979, $p<0.01$; r=–0.930, $p<0.05$, respectively) when it was present for the last 48 hr (48-h treatment) of the culture period. SRT neither demonstrated a cleavage activity on plasmid DNA nor conferred DNA protection against H_2O_2. The application of various concentrations of SRT significantly increased the TOS and oxidative stress index (OSI) in human peripheral blood lymphocytes for both the 24- and 48-h treatment periods. Morover, the increase in TOS was potent as the positive control MMC at both treatment times. However, SRT did not alter the TAS levels in either 24- or 48-h treatment periods when compared to control. In addition, exposing cells to SRT caused significant decreases in the nuclear division index at 1.25, 2.50 and 3.75 µg/mL in the 24-h and at the highest concentration (5 µg/mL) in the 48-h treatment periods. Our results suggest that SRT may have cytotoxic effect *via* oxidative stress on cultured human peripheral blood lymphocytes.

KEY WORDS: sertraline; micronucleus; pBR322; peripheral blood lymphocytes; oxidative stress; cytotoxicity

Introduction

Major depression is a leading problem in the society and is becoming increasingly more common. In the western world, major depression is the first-line psychological problem. Although it has become more common in all populations in all age groups, it occurs most frequently in young people, especially in adolescents. With this rate of increase, major depression is predicted to be the second most common disease after heart disease in 2020 (Seligman, 2006). Depression is a depressed state and activity that can affect a person's thoughts, actions, and health (APA 2013). Although the molecular mechanism of depression has not yet been fully elucidated, it is thought that the decline in the activities of some neurotransmitters leads to depression. In this context, according to the monoamine hypothesis, a decrease in the activity of brain monoamines, such as dopamine, serotonin and neuroepinephrine, leads to depression (Schildkraut, 1967).

The selective 5-HT (serotonin) reuptake inhibitors (SSRIs) reestablish the levels of 5-HT in the synaptic gap through binding the 5-HT reuptake transporter and thus averting the subsequent degradation of 5-HT. This reuptake inhibition leads to the rise of 5-HT concentrations in the synaptic gap and the concentration of 5-HT returns to the normal range. Mechanistically, this structural characteristic of SSRIs is believed to result in reduced symptoms of depression. In the presence of an SSRI agent, only a small amount of 5-HT is subjected to degradation in the synaptic gap (Rang *et al.*, 2001; Celada *et al.*, 2004).

Genotoxicity studies on SSRIs seem to indicate that each compound has unique properties due to its chemical structure and it is not feasible to reach a general judgement for the safety assessment of all SSRIs. Fluoxetine and paroxetine have been attributed as genotoxic drugs on blue mussel (*Mytilus edulis*) hemocytes (Lacaze *et al.*,

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2015). The mechanism proposed for the genotoxic effect of these drugs was the significant increase of intracellular reactive oxygen species (ROS) and oxidative stress leading to genotoxic and cytotoxic effects (Lacaze *et al.*, 2015). Based on computer modeling and energy calculations of drug/DNA complexes, the source of genotoxicity caused by citalopram was proposed to be an N-dialkyl group responsible for DNA intercalation (Synder *et al.*, 2006). Citalopram was also shown to induce genotoxicity *via* DNA strand breaks and mitotic recombination in *Aspergillus nidulans* and *Drosophila melanogaster* (Franco *et al.*, 2010; Gürbüzel *et al.*, 2012).

Sertraline (SRT) [(1S,4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine], the test substance of this study, is a widely prescribed serotonin reuptake inhibitor (MacQueen *et al.*, 2001). SRT blocks serotonin reuptake and increases serotonin stimulation of somatodendritic serotonin 1A receptor (5-HT1AR) and terminal autoreceptors in the brain. SRT treatment in patients with generalized anxiety disorder and major depression was shown to cause no DNA damage by the use of sister chromatid exchange (SCE) and chromosome aberration (CA) assays (Bozkurt *et al.*, 2004). More recently, SRT treatment did not induce DNA damage in the cells of peripheral blood, neither *in vivo* in patients with bipolar disorder nor *in vitro* at therapeutic drug concentrations in the comet assay (Andreazza *et al.*, 2007). Moreover, SRT failed to show any genotoxic effect in balancer heterozygous wings of *Drosophila melanogaster* by use of the somatic mutation and recombination test (Gürbüzel *et al.*, 2012). However, Battal *et al.* (2013) concluded that SRT administration in male Wistar albino rats increased the frequency of MN in chronic treatment (28 days), suggesting possible influence of the drug on some mechanisms of cell division.

The cytokinesis-block micronucleus cytome assay (CBMN assay) is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity (Fenech, 2007). Alterations in blood lymphocyte chromosomes are among the most widely used indicators of genotoxic exposure and a comparison of cancer risk with MN frequency is possible because MNs formed in peripheral blood lymphocytes have been validated as biomarkers of increased cancer risk (Bonassi *et al.*, 2011; Fenech *et al.*, 2011).

In the present study, to examine whether treatment of human peripheral lymphocytes with SRT leads to DNA damage, we performed CBMN cytome assay as the highly sensitive cytogenetic damage marker to detect DNA damage. Moreover, the effect of SRT in the context of DNA cleavage-protective (anti-oxidative) activity was evaluated on pBR322 plasmid. Cytotoxicity of SRT was evaluated by the nuclear division index (NDI). We also performed a quantitative approach using a novel spectrophotometric method to quantify the total oxidant status (TOS) and the total antioxidant status (TAS) in human peripheral lymphocytes exposed to SRT. The data obtained separately as the TOS and TAS were further evaluated to calculate the oxidative stress index (OSI) in blood plasma.

Materials and methods

Cukurova University Institutional Review Board was informed of the protocol to be used with human subjects and approved the protocol for the work described prior to the performance of the experiments (decision number:53; date: 13 May 2016). All healthy blood donors (n=4; two males and two females, all nonsmokers) gave informed consent before participation in this study. The healthy blood donors were not using any medication or dietary supplements throughout the study.

In vitro cytokinesis-block micronucleus (CBMN) cytome assay

Commercially available sertraline hydrochloride (SRT) (Lustral, manufactured by Pfizer) was used as the test substance for the *in vitro* tests. The genotoxic effect of SRT (dissolved in sterile bidistilled water) in human peripheral lymphocytes was studied at 4 different concentrations (1.25, 2.5, 3.75 and 5 μg/mL) (roughly 6.5–26 times higher than the maximum plasma level [0.19 μg/mL] of SRT achieved in patients receiving this drug) and 2 different exposure periods (24 or 48 hr). These concentrations were selected based on the previous range-finding studies which showed that the viability (mitotic index) in the highest concentration group (5 μg/mL) was reduced approximately by 50% as compared to that of control. The concentration-range-finding methodology was adapted from the OECD Guideline 487, in which at least three analysable test concentrations should be evaluated. However, to study the concentration-response relation in detail, we selected four closely spaced concentrations of SRT to be able to obtain better concentration-response data.

For the analysis of MN in binucleated lymphocytes, 0.2 mL of fresh whole blood (1/10 heparinized) was used to establish the cultures and the cultures were incubated for 68 h. The cells were treated with 1.25, 2.5, 3.75 and 5 μg/mL SRT for 24- and 48-h treatment periods. To block cytokinesis, cytochalasin B (Sigma, C6762) was added at 44 h of the incubation at a final concentration of 6 μg/mL. After an additional 24-h incubation at 37 °C, cells were initially harvested by centrifugation at 1,200 rpm for 15 min. Then, the supernatant discarded, cells were treated with prewarmed 0.4% KCl (37 °C) as the hypotonic solution and directly centrifuged (with the exception of a 5 min hypotonic treatment step). After centrifugation, the cells were fixed with cold fixative (1/5/6: glacial acetic acid/methanol/0.9% NaCl isotonic solution) for 20 min. Then, they were fixed two times with another cold fixative (1/5: glacial acetic acid/methanol) for 15 min. After each fixation process, the cells were centrifuged at 1200 rpm for 15 min. Later, the fixed cells were spread on cold glass slides, dried at room temperature and the slides were stained with 5% Giemsa (Kirsch-Volders *et al.*, 2003; Rothfuss *et al.*, 2000). In all subjects, 1,000 binucleated lymphocytes were scored from each donor (4,000 binucleated cells were scored per concentration). A total of 1,000 cells (4,000 cells for each treatment concentration) were scored to calculate the nuclear division index (NDI) for

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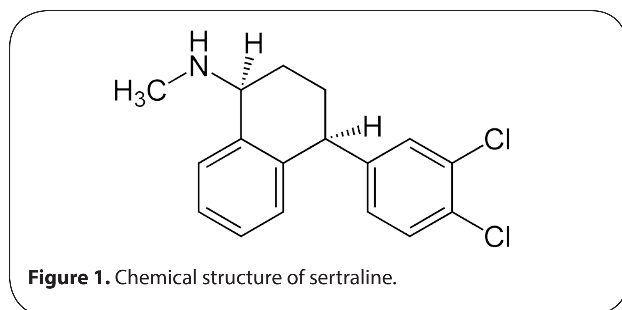

Figure 1. Chemical structure of sertraline.

the cytotoxicity of SRT using the formula: NDI = (M1) + (2 × M2) + (3 × M3) + (4 × M4)/N, where M1–M4 represent the number of cells with one to four nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells). NDI is a practical parameter for measuring the mitogenic response of lymphocytes and general cytotoxic effects of agents examined in the assay (Figure 1) (Fenech, 2007).

DNA cleavage activity and DNA damage protection potential

DNA is a main target of attack by cellular oxidant species and its damage can lead to mutation. A plasmid is a double-stranded small circular DNA molecule that is extrachromosomal and can replicate independently. Plasmids can appear in different forms.The prominent forms of monomeric plasmids are the supercoiled (SC), the open circular (OC) form (also called "nicked form" or relaxed form, obtained by digestion of one of the strands of double strand), and the linear form (LIN), typically obtained by digestion of both strands of plasmid. In the SC form, the plasmid migrates more rapidly than OC and LIN forms. These conformations, in order of electrophoretic mobility from slowest to fastest, are OC DNA, LIN DNA, and SC DNA (Timocin *et al.*, 2017; Fernandez *et al.*, 2011; Simandan *et al.*, 1998; Suksomtip & Pongsamart, 2008). DNA cleavage and protection activities of SRT were evaluated on pBR322 plasmid DNA. DNA cleavage activity was investigated with four concentrations of SRT and DNA protection activity was assayed with four concentrations of SRT in the presence of 35% H_2O_2. In summary, the experiments were performed in a microcentrifuge tube containing 3 μL of pBR322 plasmid DNA and 5 μL of SRT that was dissolved in ddH_2O. This amount (8 μL) was completed to 10 μL with ddH_2O. In addition, untreated pBR322 plasmid DNA (3 μL) as a control was also used. For DNA cleavage activity, plasmid DNA (172 ng/μL) was treated with SRT for 5 min. The plasmid DNA (pBR322) was treated with the combination (SRT + 35% H_2O_2) for 5 min at room temperature to assess possible DNA protective effect. A positive control group (plasmid DNA + 35% H_2O_2 + dH_2O) was also included in the gel. After treatments, 2 μL loading dye was added and the reactions were loaded on 1% agarose gel. Electrophoresis was performed with 100 V for 120 min in 0.5 × TBE buffer. The gels were stained with EtBr and visualized with Vilber Lourmat gel imaging system.

Plasma TAS and TOS Measurement

Blood plasma samples (2 mL) were collected from the four healthy participant donors for measurement of serum TOS and TAS. The plasma samples were immediately collected from supernatants of centrifuged whole blood cultures at the end of the cell culture period (72-hr), stored at −80° C and analyzed within 1 month.

Reactive oxygen species (ROS) are formed in metabolic and physiological processes, and desruptive oxidation reactions may occur in organisms that remove them *via* enzymatic and non-enzymatic antioxidative mechanisms. Under certain conditions, the oxidant-antioxidant balance in the cell shows an increase in the direction of the oxidant and a decrease in antioxidant, and this imbalance can not be restored by the cell. This oxidative damage causes about 100 diseases (Halliwell & Gutteridge, 2000).

The total oxidant status (TOS) of SRT-treated human peripheral blood lymphocytes was measured using automated colorimetry method of Erel. Oxidants present in the sample oxidize the ferrous ion–chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide. The results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Equiv./L) (Erel, 2004).

The TOS was calculated following the principle: 500 μL Reagent 1 (Assay Buffer) placed in cell and 75 μL of the prepared standard (or sample) was added. The initial absorbance was read at 530 nm for the first absorbance point. Then, 25 μL of Reagent 2 (Prochromogen solution) was added to the cell and incubated 5 min at room temperature. The absorbance was read at a second time at 530 nm. The results were calculated using the following formula:

$$\text{Result} = (\Delta\text{AbsSample}/\Delta\text{AbsStd}^2) \times 20\ (\text{Std}^2\ \text{Value}),$$

where Δsample Absorbance = (Second Absorbance of Sample – First Absorbance of Sample); Δabsorbance Standard 2 = (Second Absorbance of Std 2 – First Absorbance of Std 2); Standard2 Value = 20 μmol H_2O_2 Equiv./L; Std^2: (Stock Stabilized Standard Solution (SSSS)] (800 mM H_2O_2 Equiv./L).

Antioxidant molecules prevent or inhibit harmful reactions caused by oxidative damage (Young & Woodside, 2001). Because the measurement of different antioxidant molecules in plasma separately is not practical and the antioxidant effects are additive, the total antioxidant capacity of a sample is measured, and this is called total antioxidant status (TAS) (Erel, 2004). In this study, The TAS of SRT-treated plasma was measured using automated colorimetry method of Erel.

For the analysis of TAS, 500 μL of Reagent 1 (Assay Buffer) was placed in cell and 30 μL standard (or sample) was added. The initial absorbance was read at 660 nm for the first absorbance point. Then, 75 μL Reagent 2 (Colored

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ABTS Radical Solution) was added to the cell and incubated 5 min at room temperature. The absorbance was read for the second time at 660 nm. The results were expressed using the following formula:

$$[(\Delta \text{Abs Std}^1) - (\Delta \text{Abs Sample})]/[(\Delta \text{Abs Std}^1) - (\Delta \text{Abs Std}^2)],$$

where Δ Absorbance Standard^1=(Second Absorbance of Std^1 – First Absorbance of Std^1); Δ Absorbance Standard^2 = (Second Absorbance of Std2 – First Absorbance of Std^2); Δ Sample Absorbance = (Second Absorbance of Sample – First Absorbance of Sample); Std1: 0.0 mmol Trolox Equiv./L; Std2: 1.0 mmol Trolox Equiv./L.

Oxidative Stress Index (OSI)

The ratio of TOS to TAS was accepted as the oxidative stress index (OSI) (Harma *et al.*, 2003; Kösecik *et al.*, 2005; Yumru *et al.*, 2009). OSI value was calculated according to the following formula:

$$\text{OSI} = \text{TOS/TAS}$$

Statistics

Values of the control, positive control and the SRT-exposed groups were expressed as the mean (±SE) from four separate experiments. The comparisons between the control, positive control and the SRT-exposed groups were performed using One-Way ANOVA (LSD test) at $p \le 0.05$. Concentration-response relationships were determined from the correlation and regression coefficients for NDI, MN, MNBN, TOS, TAS and OSI.

Results

Table 1 shows the effect of various concentrations of SRT on the formation of micronuclei (MN) in human lymphocytes. SRT was unable to induce a statistically significant increase in the formation of MN when compared with the control for 24-h and 48-h treatment periods. Interestingly at 48-h culture period, SRT significantly decreased the %MN in a concentration-dependent ($r=-0.979$, $p<0.01$) manner (Figure 2). Increasing SRT concentrations did not cause a significant increase in the percentage of the binuclear cells with micronuclei (%MNBN) for 24- and 48-h treatment periods. On the other hand, similar to the concentration-related decrease in the %MN, SRT also induced a significant concentration-dependent decrease in the %MNBN ($r=-0.930$, $p<0.05$) when it was present for the last 48 hr (48-h treatment) of the culture period (Figure 3).

DNA cleavage activity of SRT was assessed by relaxation of supercoiled circular form of pBR322 plasmid DNA (SC DNA) into the nicked open circular (OC DNA) and linear (LIN DNA) form. If one strand of SC DNA is cleaved, the supercoil form will relax and produce slower moving OC DNA. If both strands of SC DNA are cleaved, a linear form (LIN DNA) will occur and migrate between SC DNA and OC DNA. Figure 3 depicts agarose gel electrophoresis patterns of pBR322 DNA after incubation with SRT for 5 min. Compared with control (DNA control, lane 1), the banding patterns of Lanes 3–6, demonstrate that no observable DNA cleavage occurs when incubated with four different concentrations of SRT, which is indicative of the inability of SRT to damage pBR322 DNA. Figure 5 demonstrates the DNA damage protection potential of four different concentrations of SRT. Hydrogen peroxide treatment of plasmid DNA (lane 2) resulted in the cleavage of linear DNA (LIN DNA) to faint and low molecular weight bands between OC and SC DNA. However, SRT could not show a protection potential against the DNA breaking effect of H_2O_2 on LIN DNA (lanes 3–6).

Plasma total oxidant status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) values of control and SRT-treated human peripheral blood lymphocytes are shown in Table 2. TOS were found to increase significantly in both 24- (3.75 and 5 μg/mL) and 48-h (1.25, 2.5, 3.75 and 5 μg/mL) treatment periods when compared to control. Moreover, the increase in TOS observed in 48-h treatment period showed no significant difference when compared to positive contol (Mitomycin C), indicating that SRT itself was as potent as MMC in inducing the formation of various oxidant species in human peripheral blood lymphocytes (Table 2). However, addition of SRT in human peripheral blood culture did not increase TAS in either 24- or 48-h treatment periods when compared to control. As a result of the increase in TOS, addition of SRT into human peripheral blood cultures significantly increased the OSI in 24- (3.75 and 5 μg/mL) and 48-h (2.5, 3.75 and 5 μg/mL) treatment periods.

The effects of various concentrations of SRT on cell cycling kinetics in human peripheral lymphocytes have been assessed by using the nuclear division index (NDI). The NDI shows the effect of SRT on nuclear division and hence cytotoxicity. NDI was significantly reduced at the first three concentrations (1.25, 2.50 and 3.75 μg/mL) in 24-h treatment period and at the highest concentration (5 μg/mL) in 48-h treatment period compared with control (Table 1).

Discussion

Antidepressant drugs are long-term medications used by millions of patients with or without prescription. When determining benefit/risk ratio for treatment with these drugs, it should be considered that the genotoxic and carcinogenic side effects caused by these drugs can not be ignored. Brambilla *et al.* (2009), reviewing data from several genotoxicity assays, reported that in the long-term carcinogenicity tests of 33 antidepressant molecules, 17 showed positive results in terms of genotoxicity or carcinogenicity.

SRT, the test substance of this study, is among the most widely prescribed SSRIs today (Sanches *et al.*., 2014). SRT is a type of SSRI antidepressant used mainly in major depression, obsessive-compulsive disorder, widespread and social anxiety disorder. SSRIs are preferred more

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Table 1. The percentage of micronucleus (MN), micronucleated binuclear (MNBN) cells and nuclear division index (NDI) in cultured human peripheral lymphocytes treated with SRT for 24- and 48-h.

Test substance	Treatment time (hr)	Treatment concentration (µg/mL)	MN±SE (%)	MNBN±SE (%)	NDI
Control	–	–	0.10±0.02	0.07±0.01	1.32±0.05
MM	24	0.25	0.42±0.11*	0.37±0.10*	1.13±0.02*
SRT	24	1.25	0.11±0.02b_2	0.10±0.02 b_2	1.21±0.03 a_1
SRT	24	2.50	0.18±0.07 b_1	0.16±0.07 b_1	1.18±0.02 a_2
SRT	24	3.75	0.17±0.03 b_1	0.17±0.03 b_1	1.19±0.03 a_1
SRT	24	5	0.20±0.07 b_1	0.17±0.07 b_1	1.24±0.01 b_1
MMC	48	0.25	0.42±0.20*	0.39±0.18*	1.06±0.03*
SRT	48	1.25	0.22±0.08	0.22±0.08	1.27±0.03 b_3
SRT	48	2.50	0.15±0.02	0.13±0.02	1.24±0.03 b_2
SRT	48	3.75	0.13±0.03 b_1	0.13±0.03	1.25±0.01 b_2
SRT	48	5	0.08±0.02 b1	0.08±0.02 b_1	1.18±0.02 a_2b_1

Data are expressed as the mean ± SE (n=4). * Statistically significant increase in positive control vs. control for 24 and/or 48 h (*: $p<0.05$). a Statistically significant *vs*. control, b Statistically significant *vs*. positive control. a_1b_1: $p<0.05$; a_2b_2: $p<0.01$; a_3b_3: $p<0.001$

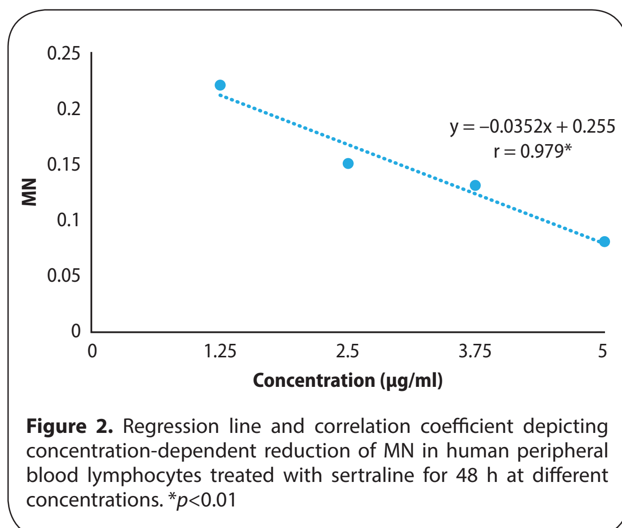

Figure 2. Regression line and correlation coefficient depicting concentration-dependent reduction of MN in human peripheral blood lymphocytes treated with sertraline for 48 h at different concentrations. *$p<0.01$

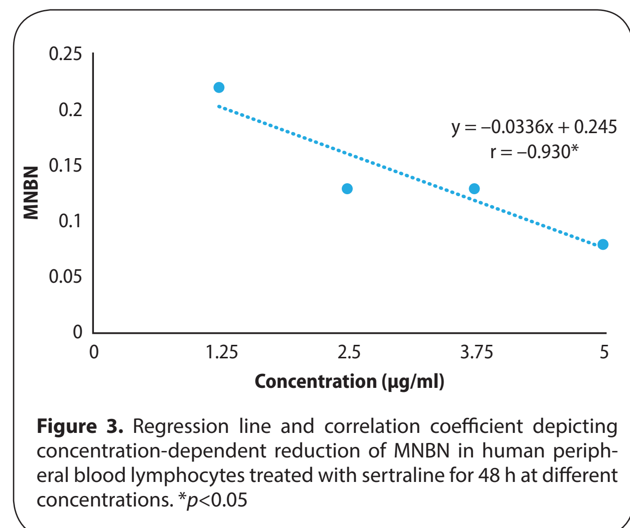

Figure 3. Regression line and correlation coefficient depicting concentration-dependent reduction of MNBN in human peripheral blood lymphocytes treated with sertraline for 48 h at different concentrations. *$p<0.05$

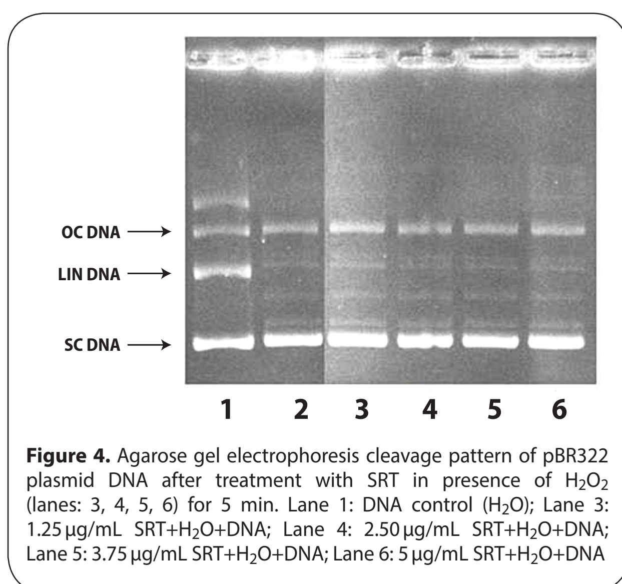

Figure 4. Agarose gel electrophoresis cleavage pattern of pBR322 plasmid DNA after treatment with SRT in presence of H_2O_2 (lanes: 3, 4, 5, 6) for 5 min. Lane 1: DNA control (H_2O); Lane 3: 1.25 µg/mL SRT+H_2O+DNA; Lane 4: 2.50 µg/mL SRT+H_2O+DNA; Lane 5: 3.75 µg/mL SRT+H_2O+DNA; Lane 6: 5 µg/mL SRT+H_2O+DNA

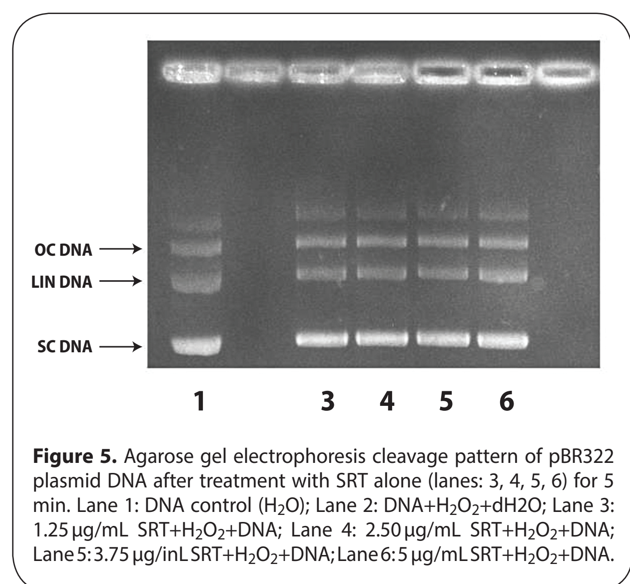

Figure 5. Agarose gel electrophoresis cleavage pattern of pBR322 plasmid DNA after treatment with SRT alone (lanes: 3, 4, 5, 6) for 5 min. Lane 1: DNA control (H_2O); Lane 2: DNA+H_2O_2+dH2O; Lane 3: 1.25 µg/mL SRT+H_2O_2+DNA; Lane 4: 2.50 µg/mL SRT+H_2O_2+DNA; Lane 5: 3.75 µg/inL SRT+H_2O_2+DNA; Lane 6: 5 µg/mL SRT+H_2O_2+DNA.

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Table 2. The levels of total oxidant status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) in cultured human blood lymphocytes treated with SRT for 24- and 48-h.

Test substance	Treatment time (hr)	Treatment concentration (μg/mL)	TOS (μmol H_2O_2 equiv./L)	TAS (mmol Trolox equiv./L)	OSI (Oxidative Stress Index)
Control	–	–	3.34±1.04	0.26±0.01	13.09±4.38
MMC	24	0.25	4.31±0.62	0.27±0.004	15.75±2.40
SRT	24	1.25	4.69±0.65	0.25±0.01	18.14±2.40
SRT	24	2.50	4.29±0.68	0.26±0.01	16.59±2.83
SRT	24	3.75	5.55±0.24 a_2	0.27±0.01	20.43±0.96 a_1
SRT	24	5	5.25±0.65 a_1	0.26±0.01	19.94±2.27 a_1
MMC	48	0.25	5.55±0.04**	0.25±0.01	22.16±1.67**
SRT	48	1.25	4.94±0.42 a_1	0.25±0.01	18.97±0.77
SRT	48	2.50	5.63±0.37 a_2	0.29±0.02	20.14±2.74 a_1
SRT	48	3.75	5.40±0.09 a_1	0.26±0.01	20.60±1.75 a_1
SRT	48	5	5.58±0.14 a_2	0.26±0.004	19.88±0.96 a_1

Data are expressed as the mean ± SE (n=4). ** Statistically significant increase in positive control vs. control for 24 and/or 48 h (**: $p<0.01$), a Statistically significant vs. control, b Statistically significant vs. positive control. a_1b_1: $p<0.05$; a_2b_2: $p<0.01$; a_3b_3: $p<0.001$.

often than older generation antidepressants because of the low side effects. However, some studies in the literature indicate that members of this class of antidepressants exert genotoxic/carcinogenic effects.

In this study, we evaluated the genotoxic potential of SRT using cytokinesis-blocked MN cytome assay. To the best of our knowledge, this is the first study to evaluate the *in vitro* genotoxic effect of SRT in cultured human peripheral lymphocytes. We used the NDI as indicator of cytotoxicity to determine the effects of SRT on the nuclear division of lymphocytes. Moreover, a quantitative approach using a novel spectrophotometric method was undertaken to quantify the amount of total antioxidants and oxidants in human peripheral lymphocytes exposed to SRT. The data obtained separately as the total antioxidant and oxidant status were further evaluated to calculate the oxidative stress index (OSI) in human lymphocytes.

In this study, SRT was unable to induce the formation of MN in human peripheral lymphocytes for 24- and 48-h treatment periods. Neither was SRT demonstrated a cleavage activity on plasmid DNA nor conferred DNA protection against H_2O_2. In accordance with our findings, SRT was shown to have no indication of clastogenic hazard in patients with generalized anxiety disorder and major depression (Bozkurt *et al.*, 2004). Bozkurt *et al.* concluded that the increased SCE frequency in patients was based on psychogenic stress rather than SRT treatment. Andreazza *et al.* (2007) reported that the DNA damage observed in peripheral leukocytes of patients in the comet assay was correlated with the symptoms of depression and mania, but not with SRT treatment. Furthermore, SRT, though not citalopram, did not show any genotoxic effect in balancer heterozygous wings of D. melanogaster (Gürbüzel *et al.*, 2012). However, Battal *et al.* (2013) reported that SRT induced a significant increase in the frequency of MN (in chronic treatments) in peripheral blood of Wistar albino rats in the CBMN assay.

The reason for the apparent discordance that SRT could not induce DNA damage in our study and in the comet assay (Bozkurt *et al.*, 2004; Andreazza *et al.*, 2007; Gürbüzel *et al.*, 2012), but apparently induced DNA damage in the *in vivo* CBMN assay (Battal *et al.*, 2013) is not clear but negative comet assay results versus positive MN results should be interpreted carefully. Comet assay measures primary DNA damage, which is usually completely repaired within a few hours by the DNA repair mechanisms of the cell. If the comet assay is performed a few hours after the induction of DNA damage, comet data could be negative because of the effective repair of DNA damage. Improperly repaired damage can cause MN in cells undergoing second mitotic division (M2). The data can be interpreted as the absence of induction of primary DNA damage and as a negative comet assay result (Wiedemann & Schutz, 2008). On the other hand, high-dose SRT administration has been shown to enhance oxidative stress, lipid peroxidation (Battal *et al.*, 2014) and the oxidative stress caused by SRT leads to mitochondrial dysfunction in eukaryotic cells (Li *et al.*, 2012). Furthermore, it was shown that ROS inducing chemicals could induce secondary DNA damage (Jeong and Swenberg, 2004). Thus, we are of the opinion that high MN frequency observed in the study of Battal *et al.* (2013) may be secondary DNA damage caused by oxidative cell injury resulting from long-term chronic administration of SRT in rats.

The present study clearly demonstrates a SRT-induced cytotoxicity in human peripheral lymphocytes as revealed by significant decreases in the NDI. Interestingly, at a 48-h culture period, SRT induced a significant concentration-dependent decrease in the percentage of MN and MNBN. We are of the opinion that this effect of SRT could be explained by the suppression of nuclear division due to oxidative stress. For the formation of micronucleus, binuclear cells that have undergone only one nuclear division must be present. The decrease in the number of binuclear

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cells (due to decrease in nuclear division) was determined as a decrease of MN in our study. At the molecular level, this effect on lymphocyte cell division was shown to be related to the suppressive effect of SRT on expression of genes (Cdc6) and proteins (STAT3 and COX2) involved in cell proliferation (Taler *et al.*, 2007). SRT was also shown to inhibit translation by altering the localization of eIF4E and increasing eIF2α phosphorylation. In addition, these effects were followed by the downregulation of mTOR signaling pathway in a REDD1-dependent manner (Lin *et al.*, 2010).

The results obtained with SRT on the cellular antioxidant and oxidant status in our study showed that SRT is a potent inducer of cellular oxidant species as well as oxidative stress. Although a measurement of the total oxidant status (but not different oxidant molecules separately) performed in this study showed that our results on the cytotoxicity of SRT are in apparent concordance with the study of Chen *et al.* (2014). In that study, SRT decreased the cell viability and induced apoptosis in human hepatoma cell line HepG2 through the TNFα-MAP4K4-JNK pathway. These data clearly suggest that SRT can pose cytotoxic risk even at very low concentrations *in vitro*. All SSRI antidepressants carry apolar aromatic ring systems in their molecular structures. Although we did not perform a study in this context, we propose that the oxidative stress induced by SRT in our study may be correlated to its ability to easily cross and damage biological membranes (both cellular and mitochondrial). Consistent with our argument, SRT has been also shown to diffuse passively through lysosomal membranes and accumulate in the organelle (lysosomotropy) *via* a proton-trap mechanism (Daniel *et al.*, 2001). This feature of SRT leads to damage to the lysosomal membrane through increased ROS, lipid peroxidation, and activation of cathepsins (Dielschneider *et al.* 2017). In conclusion, SRT and other cationic amphiphilic drugs demonstrate antiproliferative effect by ion trapping, vacuolar alterations and these effects are inversely correlated with their lipophilicity (Parks & Marceau, 2016).

Conclusion

In this study, oxidant, antioxidant, genotoxic and DNA cleavage-damage protection potential of SRT were determined. In general, SRT was unable to induce micronuclei and did not demonstrate DNA cleavage activity on pBR322 DNA. Neither was SRT potent enough to reverse the DNA cleavage activity of H_2O_2. Our results are in accordance with the study of Synder *et al.* (2006) who reported that unlike genotoxic citalopram, SRT does not have the N-dialkyl group that enables the molecule for possible DNA intercalation. However, SRT exerted cytotoxic effect by decreasing the NDI and increased the oxidative stress index in human peripheral lymphocytes. Previous studies have also confirmed that the use of SRT is associated with increased oxidative stress, lipid peroxidation (Chung *et al.*, 2013; Battal *et al.*, 2014), mitochondrial and lysosomal dysfunction (Li *et al.*, 2012; Dielschneider *et al.* 2017). Therefore, it is essential that antidepressant drugs should be tested not only for their genotoxic potential but also for their ability to disturb mitochondrial and lysosomal processes which lead to cytotoxicity and thus to provide a deeper understanding of the potential risks related to treatment in patients with depression.

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REFERENCES

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. Arlington, VA: American Psychiatric Publishing.

Andreazza AC, Frey BN, Erdtmann B, Salvador M, Rombaldi F, Santin A, Gonçalves CA and Kapczinski F. (2007). DNA damage in bipolar disorder. *Psychiatry Res* **153**: 27–32.

Battal D, Aktas A, Sungur MA, Kadıoğlu E, Eker ED, Sahin NO and Saygı S. (2013). In vivo genotoxicity assessment of sertraline by using alkaline comet assay and the cytokinesis-block micronucleus assay. *Basic Clin Pharmacol Toxicol* **113**: 339–46.

Battal D, Yalin S, Eker ED, Aktas A, Sahin NO, Cebo M and Berkoz M. (2014). Possible role of selective serotonin reuptake inhibitor sertraline on oxidative stress responses. *Eur Rev Med Pharmacol Sci* **18**: 477–484.

Bonassi S, El-Zein R, Bolognesi C and Fenech M. (2011). Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies. *Mutagenesis* **26**: 93–100.

Bozkurt G, Abay E, Ates I, Karabogaz G, Ture M, Savran FO, Palanduz S, Temocin K and Algunes C. (2004). Clastogenicity of selective serotonin-reuptake inhibitors. *Mutat Res* **558**: 137–44.

Brambilla G, Mattioli F and Martelli A. (2009). Genotoxic and carcinogenic effects of antipsychotics and antidepressants. *Toxicology* **261**: 77–88.

Celada P, Puig MV, Amargós-Bosch M, Adell A and Artigas F. (2004). The therapeutic role of 5-HT1A and 5-HT2A receptors in depression. *J Psychiatry Neurosci* **29**: 252.

Chen S, Xuan J, Wan L, Lin H, Couch L, Mei N, Dobrovolsky VN and Guo L. (2013). Sertraline, an antidepressant, induces apoptosis in hepatic cells through the mitogen-activated protein kinase pathway. *Toxicol Sci* **137**: 404–415.

Chung CP, Schmidt D, Stein CM, Morrow JD and Salomon RM. (2013). Increased oxidative stress in patients with depression and its relationship to treatment. *Psychiatry Res* **206**: 213–216.

Daniel WA, Wójcikowski J and Pałucha A. (2001). Intracellular distribution of psychotropic drugs in the grey and white matter of the brain: the role of lysosomal trapping. *Br J Pharmacol* **134**: 807–814.

Dielschneider RF, Henson ES and Gibson SB. (2017). Lysosomes as oxidative targets for cancer therapy. *Oxid Med Cell Longev* **2017**: 3749157.

Erel O. (2004). A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* **37**: 277–85.

Fenech M. (2007). Cytokinesis-block micronucleus cytome assay. *Nature Protocols* **2**: 1084–1104.

Fenech M, Holland N, Zeiger E, Chang WP, Burgaz S, Thomas P, Bolognesi C, Knasmueller S, Kirsch-Volders M and Bonassi S. (2011). The HUMN and HUMNxL international collaboration projects on human micronucleus assays in lymphocytes and buccal cells–past, present and future. *Mutagenesis* **26**: 239–245.

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Fernandez LL, Montes-Bayon M, Gonzalez EB, Sierra LM, Sanz-Medel A and Bettmer J. (2011). Initial studies on quantitative DNA induced oxidation by gel electrophoresis (GE)-ICP-MS. *J Anal Atom Spectrom* **26**: 195–200.

Franco CC, Castro-Prado J, Rosada LJ, Sant'Anna JR and Castro-Prado MA. (2010). Mitotic recombination: a genotoxic effect of the antidepressant citalopram in *Aspergillus nidulans*. *Exp Biol Med* **235**: 1257–1262.

Gürbüzel M, Oral E, Kızılet H, Halıcı Z and Gulec M. (2012). Genotoxic evaluation of selective serotonin-reuptake inhibitors by use of the somatic mutation and recombination test in *Drosophila melanogaster*. *Mutat Res* **748**: 17–20.

Halliwell B and Gutteridge JMC. (2000). *Free radicals in biology and medicine*. Third ed. Oxford: Oxford Science Publications; p. 617–24.

Harma M, Harma M and Erel O. (2003). Increased oxidative stress in patients with hydatidiform mole. *Swiss Med Wkly* **133**: 563–536.

Jeong YC and Swenberg JA. (2004). Secondary DNA damage from reactive oxygen species. *Cancer Res* **64**: 175.

Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate M Jr, Kirchner S, Lorge E, Morita T, Norppa H, Surralles J, Vanhauwaert A and Wakata A. (2003). Report from the in vitro micronucleus assay working group. *Mutat Res* **540**: 153–163.

Kösecik M, Erel O, Sevinc E and Selek S. (2005). Increased oxidative stress in children exposed to passive smoking. *Int J Cardiol* **100**: 61–4.

Lacaze E, Pédelucq J, Fortier M, Brousseau P, Auffret M, Budzinski H and Fournier M. (2015). Genotoxic and immunotoxic potential effects of selected psychotropic drugs and antibiotics on blue mussel (*Mytilus edulis*) hemocytes. *Environ Pollut* **202**: 177–186.

Li Y, Couch L, Higuchi M, Fang JL and Guo L. (2012). Mitochondrial dysfunction induced by sertraline, an antidepressant agent. *Toxicol Sci* **127**: 582–591.

Lin CJ, Robert F, Sukarieh R, Michnick S and Pelletier J. (2010). The antidepressant sertraline inhibits translation initiation by curtailing mammalian target of rapamycin signaling. *Cancer Res* **70**: 3199–3208.

MacQueen G, Born L and Steiner M. (2001). The selective serotonin reuptake inhibitor sertraline: its profile and use in psychiatric disorders. *CNS Drug Rev* **7**: 1–24.

Parks A and Marceau F. (2016). Lysosomotropic cationic drugs induce cytostatic and cytotoxic effects: Role of liposolubility and autophagic flux and antagonism by cholesterol ablation. *Toxicol Appl Pharmacol* **305**: 55–65.

Rang HP, Dale MM and Ritter JM. (2001). *Pharmacology*, 4th edition. Edinburgh, UK Harcourt Publishers Ltd, 2001165–176.

Rothfuss A, Schutz P, Bochum S, Volm T, Eberhardt E, Kreirenberg R, Vogel W and Speit G. (2000). Induced micronucleus frequencies in peripheral lymphocytes as a screening test for carriers of a BRCA1 mutation in breast cancer families. *Cancer Res* **60**: 390–394.

Schildkraut JJ. (1967). The catecholamine hypothesis of affective disorders. A review of supporting evidence. *Int J Geriatr Psychiatry* **4**: 203–17.

Seligman MEP. (2006). *Learned optimism*. Vintage Books. New York. 319p.

Simandan T, Sun J and Dix TA. (1998). Oxidation of DNA bases, deoxyribonucleosides and homopolymers by peroxyl radicals. *Biochem J* **33**5: 233–240.

Snyder RD, Ewing D and Hendry LB. (2006). DNA intercalative potential of marketed drugs testing positive in in vitro cytogenetics assays. *Mutat Res* **609**: 47–59.

Suksomtip M and Pongsamart S. (2008). Protective effect against oxidation of human low-density lipoprotein and plasmid DNA strand scission of Tamarind seed coat extract in vitro. *LWT – Food Sci Technol* **41**: 2002–2007.

Taler M, Gil-Ad I, Lomnitski L, Korov I, Baharav E, Bar M, Zolokov A and Weizman, A. (2007). Immunomodulatory effect of selective serotonin reuptake inhibitors (SSRIs) on human T lymphocyte function and gene expression. *Eur Neuropsychopharmacol* **17**: 774–780.

Timocin T, Husunet MT, Valipour E, Norizadeh Tazehkand, M, Celik R, Topaktas M and Ila H B. (2017). In vitro cytogenetic evaluation of the particular combination of flurbiprofen and roxithromycin. *Drug Chem Toxicol* **40**: 326–332.

Wiedemann PM and Schutz H. (2008). *The role of evidence in risk characterization: making sense of conflicting data*. Weinheim: Wiley-VCH

Young IS and Woodside JV. (2001). Antioxidants in health and disease. *J Clin Pathol* **54**: 176–86.

Yumru M, Savas HA, Kalenderoglu A, Bulut M, Celik H and Erel O. (2009). Oxidative imbalance in bipolar disorder subtypes: a comparative study. *Prog Neuropsychopharmacol Biol Psychiatry* **33**: 1070–4.

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ORIGINAL ARTICLE

Insights on the relationship between structure vs. toxicological activity of antibacterial rhodamine-labelled 3-hydroxy-4-pyridinone iron(III) chelators in HepG2 cells

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ABSTRACT

In the present study we investigated the *in vitro* hepatotoxicity of a set of rhodamine-labelled 3-hydroxy-4-pyridinones (3,4-HPO) that had previously demonstrated significant inhibitory effect in the intramacrophagic growth of *Mycobacterium avium*. Our aim was to establish a correspondence between the molecular structure and the *in vitro* toxicological activity of these compounds.
The impact of a set of bidentate (MRB2, MRB7, MRB8, and MRB9) and hexadentate (MRH7, MRH8, and MRH10) chelators on cellular metabolic competence and membrane integrity was investigated in HepG2 cells.
Our findings indicate that: a) hexadentate chelators are more cytotoxic than parent bidentate ligands; b) disruption of cell membrane and metabolic competence only occurred after 5 days, at the highest concentrations tested; c) strict correlation between bacteriostatic activity and *in vitro* toxicity was observed, which seems to be directly dependent on the size of the molecule and on the hydrophilic/lipophilic balance; d) among the set of bidentate ligands, carboxyrhodamine derivatives (amide linker) presented lower detrimental effects, when compared with rhodamine B isothiocyanate chelators (thiourea linker); e) contrarily, for the hexadentate series, rhodamine B isothiocyanate derivatives are less cytotoxic to HepG2 cells than carboxyrhodamine molecules; and f) for all compounds tested, when the substituents of the nitrogen atom were switched from ethyl to methyl, an increment of toxicity was observed.
Overall, all chelators seem to display suitable in vitro toxicological potential to combat fast grow bacteria. According to their *in vitro* pharmacological: toxicological potential ratio, MRH7 and MRH8 may be considered as the most suitable compounds to undergo further pre-clinical development studies.

KEY WORDS: iron chelator; 3-hydroxy-4-pyridinone (3,4-HPO); rhodamine; *in vitro* toxicity; HepG2 cells

Introduction

Considering the alarming emergence and spread of new forms of antimicrobial resistance, the development of new strategies to fight bacterial infections remains crucial to provide physicians with effective tools for bringing these pathogens under control. In this regard, our research group has been engaged in efforts to design novel effective antibiotics against mycobacterial species, specifically *Mycobacterium avium* (Fernandes *et al.*, 2010, Nunes *et al.*, 2010, Moniz *et al.*, 2013, Moniz *et al.*, 2015).

Some studies have shown a link between *M. avium* infection and increased iron deposition in tissues of infected patients (al-Khafaji *et al.*, 1997); this essential element impairs macrophage innate immunity and, consequently, favours the growth and virulence of the opportunist agent (Silva-Gomes *et al.*, 2013). An additional factor that supports the importance of this element in mycobacterial infection is that iron uptake is mediated by high-affinity chelating structures produced

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by the pathogen, the siderophores mycobactins (cell wall-associated) and carboxymycobactins (released into the extracellular medium) (Thompson *et al.,* 2006). Since iron is essential for mycobacterial growth and survival (Silva-Gomes *et al.,* 2013), iron chelation therapy, as means of removal of metal available for mycobacteria metabolism, is worth considering. Coherently, iron deprivation has proven to prevent *M. avium* proliferation in mice, although few different iron chelators have demonstrated only marginal *in vitro* mycobacteriostatic activity in macrophages (pathogen natural host cells) (Gomes *et al.,* 1999, Cronje & Bornman, 2005).

Following this research line, several 3-hydroxy-4-pyridinones (3,4-HPO) functionalized with different rhodamine moieties were synthesised (Figure 1), and their antibacterial activity against *M. avium* infection was further evaluated (Fernandes *et al.,* 2010, Nunes *et al.,* 2010, Moniz *et al.,* 2013). Carboxytetramethylrhodamine-labelled chelators (MRB8 and MRH8) were deemed capable of limiting iron supply and restricting intramacrophagic growth of *M. avium*, although in a lesser extent than rhodamine B isothiocyanate-labelled chelators (MRB7 and MRH7) (Figure 1; Table 1) (Moniz *et al.,* 2013). Since the Fe(III)-chelating competence was similar for all testing agents, we hypothesised that the pharmacological potential of these compounds was also reliant on their ability to reach relevant intracellular targets, namely by crossing membranes to reach the cytosol of the host cell or also the phagosomes. This capacity of the chelators to permeate biological membranes is dependent on a) the substituents of the amino groups of the xanthene ring of rhodamine; and b) the type of linkage between rhodamine and the chelating unit (Nunes *et al.,* 2010, Moniz *et al.,* 2016b, Moniz *et al.,* 2017).

Although some data clarifying the molecular features that are crucial for the antimycobacterial activity of those chelators already exist, no information is available regarding their eventual toxicity. Parallel to the assessment of pharmacological efficacy, the evaluation of toxic profile of new compounds is of great relevance in the process of developing new therapeutic agents, as the detrimental effects of drugs may severely compromise their clinical application. Thus, in order to evaluate the potential toxicity elicited by rhodamine-labelled chelators that have formerly demonstrated antimicrobial properties (Figure 1; Table 1) and to elucidate the main molecular features underlying those detrimental effects, we investigated the impact of several ligands on cellular metabolic competence and on cytoplasmic membrane integrity, using the human hepatoma cell line HepG2.

Materials and methods

Chemicals

The structure of the bidentate and hexadentate chelators used in the present study are shown in Figure 1. The chelators MRB2, MRB7, MRB8, MRB9, MRH7, MRH8, and MRH10 were previously synthesized by our research group. Their synthesis and characterization are described elsewhere (Nunes *et al.,* 2010, Moniz *et al.,* 2013, Moniz *et al.,* 2015, Moniz *et al.,* 2016a). Unless stated otherwise, all the cell culture reagents were purchased from Gibco (Alfagene, Lisbon, Portugal) and all other chemicals from Sigma-Aldrich (Lisbon, Portugal).

HepG2 cell culture routine

The human hepatoma HepG2 cell line was generously given by Prof. Ricardo Dinis-Oliveira (CESPU, Instituto Superior de Ciências da Saúde – Norte, Gandra, Portugal). HepG2 cells were routinely maintained in 75 cm^2 canted-neck tissue culture flasks Corning® (VWR, Lisbon, Portugal) and cultured as monolayer in cell culture medium, *i.e.* DMEM, high glucose, GlutaMAX™ (Sigma-Aldrich, Lisbon, Portugal) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic (penicillin 5000 U/mL + streptomycin 10000 µg/mL), and 1% fungizone (250 mg/mL amphotericin B), in a humidified incubator with a 5% CO_2 atmosphere, at 37 °C. Cells were sub-cultured at approximately 70–80% confluence, over a maximum of 10 passages (to avoid accumulation of genetic mutations during replicative process). To promote cells detachment, the cells were washed with Hank's balanced salt solution (HBSS) after medium removal, and

Table 1. Structural features, antibacterial activity (Moniz *et al.,* 2013, Moniz *et al.,* 2015) and interaction with biological membranes (Coimbra *et al.,* 2014, Moniz *et al.,* 2016b, Moniz *et al.,* 2017) of the rhodamine-labelled 3-hydroxy-4-pyridinone iron(III) chelators.

Chelator	Structure features				Antibacterial activity against *Mycobacterium avium*	Membrane interaction
	Denticity	Type of fluorophore	*N*-substituents	Linker		
MRB2	Bidentate	Tetraethyl sulphorhodamine B	*N*-ethyl	Sulphonamide	0	nd
MRB7	Bidentate	Tetraethyl rhodamine B isothiocyanate	*N*-ethyl	Thiourea	++++	++
MRB8	Bidentate	Tetramethyl carboxyrhodamine	*N*-methyl	Amide	++	+
MRB9	Bidentate	Tetraethyl carboxyrhodamine	*N*-ethyl	Amide	++	nd
MRH7	Hexadentate	Tetraethyl rhodamine B isothiocyanate	*N*-ethyl	Thiourea	++++	++
MRH8	Hexadentate	Tetramethyl carboxyrhodamine	*N*-methyl	Amide	+++	+
MRH10	Hexadentate	Tetramethyl rhodamine B isothiocyanate	*N*-methyl	Thiourea	+++	nd

0 – no effect; + low; ++ moderate; +++ high; ++++ very high; nd – not determined (no detailed studies have been performed for these chelators regarding their capacity to interact with membranes)

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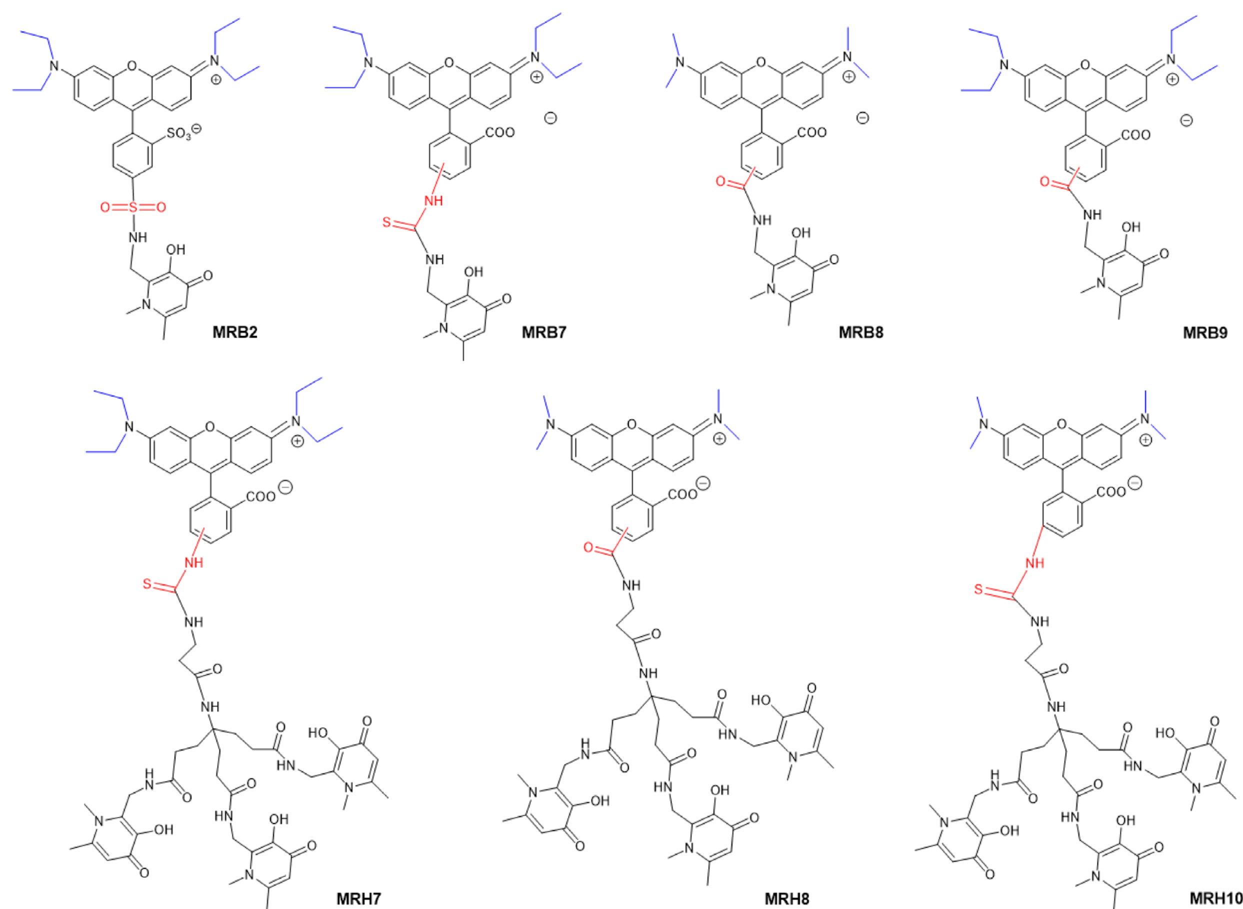

Figure 1. Structure and nomenclature of fluorescent bidentate (MRB2, MRB7, MRB8, and MRB9) and hexadentate (MRH7, MRH8, and MRH10) chelators used in the current study. In previous works, the compound MRH7 was abbreviated as CP777 (Fernandes *et al.,* 2010) and 4 (Nunes *et al.,* 2010). The molecular structure of these chelators comprise three parts: *i)* a chelating unit, *i.e.* 3-hydroxy-4-pyridinone (3,4-HPO); *ii)* a fluorophore (tetraethyl sulphorhodamine B for MRB2; tetraethyl rhodamine B isothiocyanate for MRB7 and MRH7; tetramethyl carboxyrhodamine for MRB8 and MRH8; tetraethyl carboxyrhodamine for MRB9; or tetramethyl rhodamine B isothiocyanate for MRH10); and *iii*) a linkage fragment, which is determined by the fluorophore derivative that is used in the synthesis. The chelating unit is directly coupled to the fluorophore to produce the bidentate fluorescent ligands (MRB2, MRB7, MRB8, and MRB9) or linked to a tripodal anchor to produce hexadentate chelators (MRH7, MRH8, and MRH10). The substituents on xanthene group of rhodamine are highlighted in blue. The linker between the rhodamine fluorophore and the chelating unit is highlighted in red.

added of 2 mL of 0.25% trypsin/1 mM EDTA solution. After a 5 min-incubation at 37 °C, cells were re-suspended in 10 mL complete cell culture medium and split into new flasks at appropriate ratios. For the cytotoxicity assays, cells were plated onto 96-well plates (BD Falcon, Enzifarma, Lisbon, Portugal) at a density of 5×10^4 per well (100 µL of suspension to each well) and placed in the incubator at 37 °C, for approximately 24 h, prior to the compound treatments. Peripheral wells on the plate were filled with sterile water to prevent evaporation and concentration of the test solutions (da Silva *et al.,* 2014).

Incubation of the chelators

On the day of the experiment, the medium was removed and HepG2 cells were exposed to a set of concentrations of each rhodamine-derived chelator (ranging from 1 to 40 µM and from 3 to 120 µM for hexadentate and bidentate ligands, respectively). The concentration intervals were selected on the basis of the concentrations tested in previous studies for the evaluation of the antimycobacterial effect (Moniz *et al.,* 2013, Moniz *et al.,* 2015). The concentrations tested of hexadentate chelators were three times lower than those selected for bidentate ligands; this testing design is explained by the fact that three molecules of a bidentate ligand are needed to chelate the same amount of iron(III) chelated by one molecule of a hexadentate ligand.

Stock solutions were prepared in 4% DMSO (V/V) and stored at 4 °C, whereas test dilutions were freshly prepared in complete cell culture medium on the day of the experiment. In all experiments, a solvent control (cells treated with 0.05% DMSO, V/V) was included to mimic the maximum concentration of DMSO present in the treatments. A positive control consisting of 1% Triton X-100 (V/V) and a negative control (complete cell culture medium without treatments) were also included. Each treatment condition

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was tested in triplicates by adding 250 µL of solution to each well of the 96-well plate. Cells were then incubated at 37 °C, for 5 days. No significant differences were observed between solvent and negative controls (data not shown; $p>0.05$, Student's unpaired *t*-test).

Evaluation of cytoplasmic membrane integrity by the LDH leakage assay

The LDH leakage assay was performed to access the membrane integrity of HepG2 cells in the presence of chelators. As LDH is a cytoplasmic oxidoreductase, its presence in the extracellular medium is indicative of alterations in membrane permeability and consequently in cell integrity.

The enzyme catalyses the reversible conversion of pyruvate to lactate, in the presence of β-nicotinamide adenine dinucleotide (β-NADH), which in turn is oxidized to NAD^+. So, after 1 day, 3 days, and 5 days of incubation with the chelators, a 10 µL aliquot of supernatant of each well was placed into a new 96-well plate. Then 40 µL of 0.05 M potassium phosphate buffer (pH 7.4, KH_2PO_4, Merck) and 200 µL of 0.15 mg/mL β-NADH were added. Immediately before the absorbance reading, 25 µL of 2.5 mg/mL sodium pyruvate were pipetted into each well to start the reaction. The β-NADH and sodium pyruvate solutions were freshly prepared in buffer solution. The oxidation of NADH to NAD^+ was followed by measuring the absorbance at 340 nm (Amit *et al.,* 2014), every 16 seconds, for 3 min, using an automatic plate reader Power Wave X™ (BioTek Instruments, Inc.) in a kinetic photometric mode. Since the absorbance range of the 3,4-HPO chelators does not overlap with that for NAD^+, there is no possibility of interference in the spectrophotometric assay. Data were scaled between positive (100%) and negative controls (0%) and results were graphically presented as percentage of extracellular LDH *versus* chelator concentration (µM).

Evaluation of cellular functioning by the MTT reduction assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay is a colorimetric test used to measure the activity of the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidoreductases that reduce the soluble yellow MTT into purple insoluble crystals of formazan. Cellular NADPH-dependent oxidoreductase activity is an indicator of general viability, as the redox state is a key parameter of cellular physiology, desegregating evidence on the cell catabolic efficiency, antioxidant defence, and biosynthetic potential. Since NADPH-dependent enzymes are largely ubiquitous in the cytosolic compartment, the amount of purple formazan produced by cells treated with the chelators was compared with the amount of formazan produced by untreated controls to assess the ability of the compound to change the normal metabolic competence of the cell. After 5 days of incubation at 37 °C, the medium was removed and 100 µL 0.5 mg/mL MTT (prepared in HBSS) were added to the attached cells. The plates were incubated at 37 °C, for 90 min. Then, the MTT solution was aspirated and the formed intracellular formazan crystals were dissolved with 100 µL DMSO. The plates were shaken for 15 min and, as MTT is photosensitive, all steps of the procedure were executed under light protection. The absorbance was recorded at 550 nm, using a multi-well plate reader BioTek Synergy™ HT (BioTek Instruments, Inc.) (Dias da Silva *et al.,* 2013, da Silva *et al.,* 2014). Data were scaled between negative (100%) and positive controls (0%) and results were graphically presented as percentage of MTT reduction *versus* chelator concentration (µM).

Statistical analysis

The cytotoxicity data from the MTT reduction and LDH leakage assays were obtained from four independent experiments. Eight increasing test concentrations of each chelator were tested in three replicates, within each experiment. To reduce variability between experiments, data were normalized plate-by-plate by negative and positive controls, as previously described (Rajapakse *et al.,* 2004, Dias da Silva *et al.,* 2013, da Silva *et al.,* 2014). The results are presented as mean ± standard error of the mean (SEM). Normality of data distribution was assessed by the Kolmogorov-Smirnov, D'Agostino & Pearson omnibus, and Shapiro-Wilk normality tests. Statistical comparisons were performed by Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. The solvent and negative control values (raw data) were compared by the Student's unpaired *t*-test and differences were considered insignificant (data not shown). Nonlinear regression analysis of all four bidentate chelators was carried out using the Hill equation. All of the nonlinear regression models describe sigmoidal concentration–response relationships. Data obtained were analysed using the software Graph Pad Prism version 6.0. Significance was accepted at $p<0.05$.

Results

Results obtained in LDH leakage assay show that disruption of cytoplasmic membrane increases over time when hepatocytes are incubated with the chelators tested. Accordingly, no effects were observed after one day (Figures 2 and 3, $p>0.05$). After three days, a tendency for chelator-induced disturbance of cell integrity was observed, however this observed augment of membrane disruption had no statistical significance ($p>0.05$). Significant differences were only detected after five days of exposure to bidentate ligand MRB8 (Figure 2, $p<0.05$; from 24.5 µM up) and to hexadentate MRH7, MRH8 and MRH10 ligands (Figure 3, $p<0.05$; from 1.5 µM up). The less cytotoxic chelators were bidentate ligands MRB2, MRB7 and MRB9, whose LDH leakage effect was not higher than 12.79±9.54%, 16.63±7.59% and 15.26±8.71%, respectively (Figure 2, $p>0.05$). With exception of MRB7, these are also the chelators displaying the lowest antibacterial activity (Moniz *et al.,* 2013, Moniz *et al.,* 2015). Results of the LDH leakage in HepG2 cells following 1, 3, and 5 day-exposures to bidentate and hexadentate chelators are presented in Figures 2 and 3, respectively.

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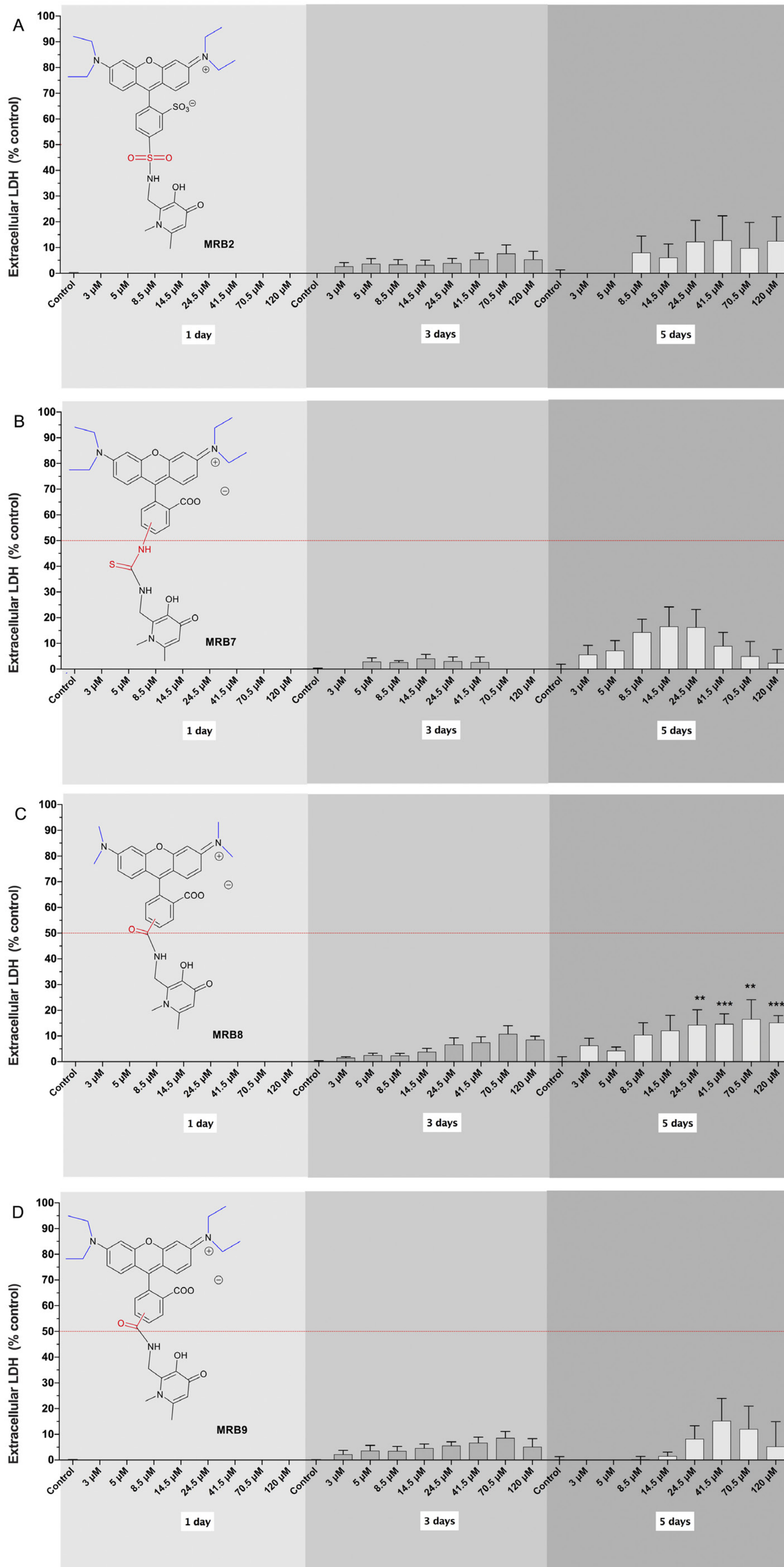

Figure 2. Cell membrane disruption assessed by the leakage of lactate dehydrogenase (LDH) to the extracellular medium, in Hep G2 cells, following exposure to bidentate chelators, *i.e.* MRB2 (A), MRB7 (B), MRB8 (C), and MRB9 (D), for 1, 3, or 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 3 to 120 µM. Data are presented as mean ± standard error of the mean (SEM) of percentage of extracellular LDH, relative to the positive controls (cells treated with 1% Triton-X100), and were obtained in four independent experiments, performed in triplicates. The dashed red line represents half of the percentage of LDH leakage obtained for the positive controls (50%). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *$p<0.05$; **$p<0.01$ and ***$p<0.001$, when compared with controls at the same time-point.

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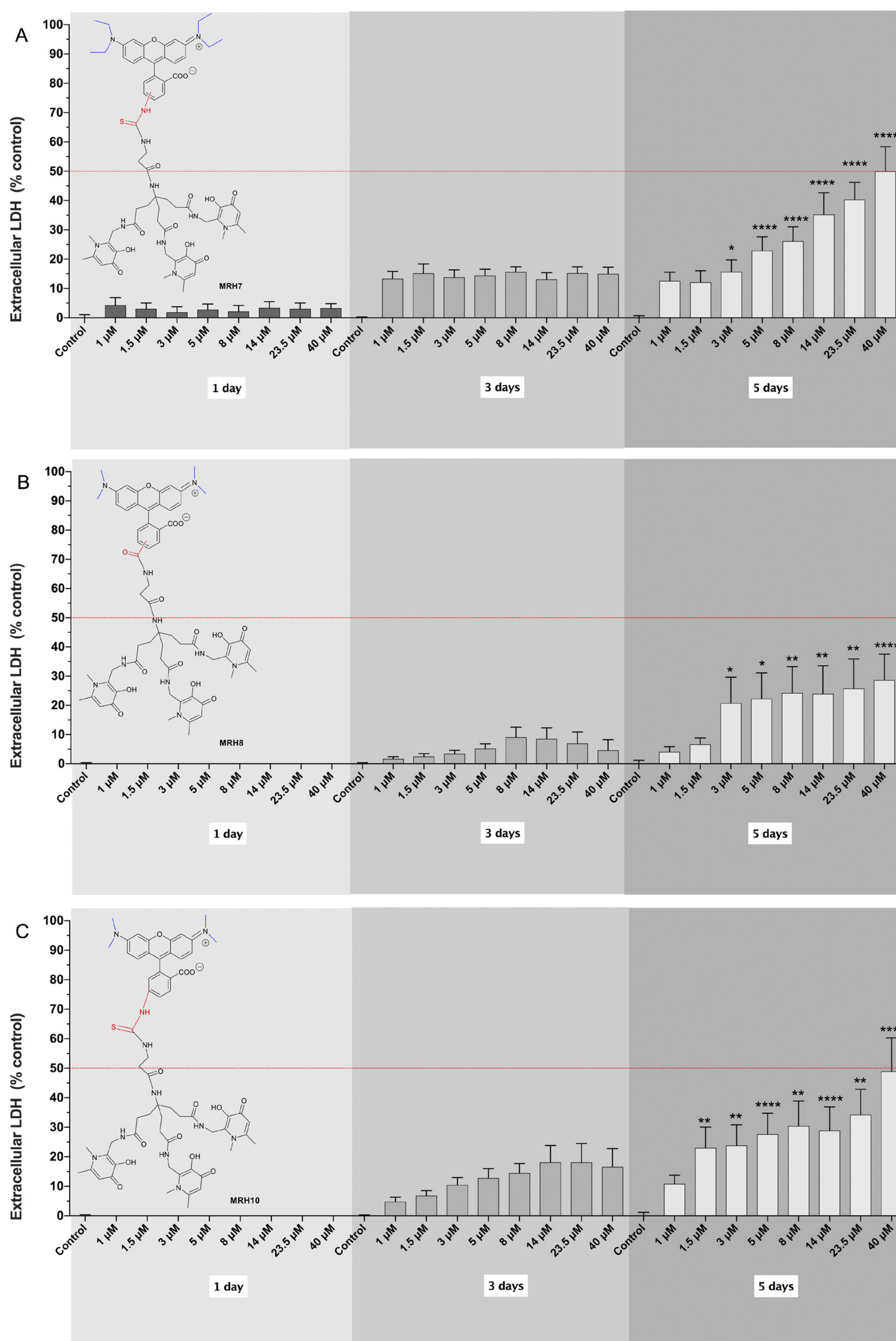

Figure 3. Cell membrane disruption assessed by the leakage of lactate dehydrogenase (LDH) to the extracellular medium, in Hep G2 cells, following exposure to hexadentate chelators, *i.e.* MRH7 (A), MRH8 (B), and MRH10 (C), for 1, 3, or 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 1 to 40 μM. Data are presented as mean ± standard error of the mean (SEM) of percentage of extracellular LDH, relative to the positive controls (cells treated with 1% Triton-X100), and were obtained in four independent experiments, performed in triplicates. The dashed red line represents half of the percentage of LDH leakage obtained for the positive controls (50%). Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, when compared with controls at the same time-point.

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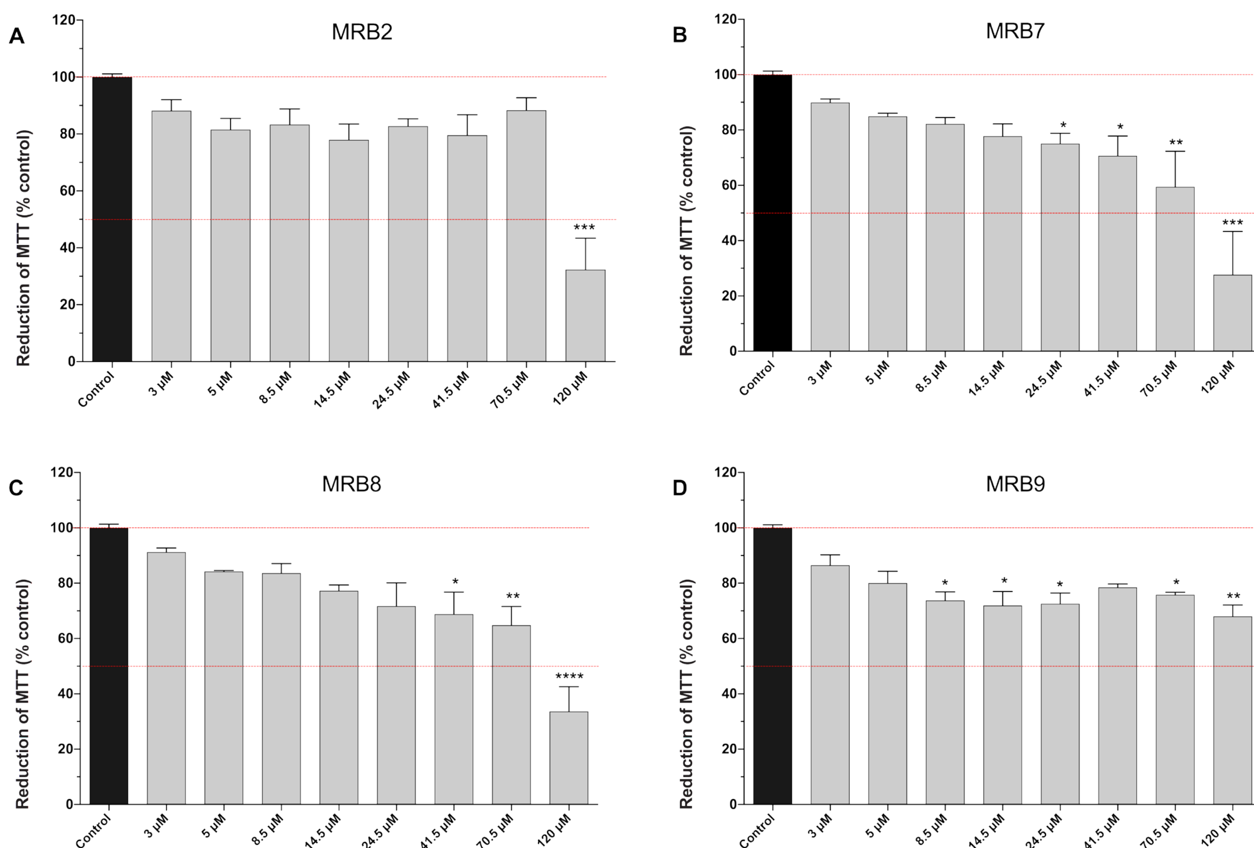

Figure 4. Cell metabolic competence assessed by the reduction of the MTT assay, following exposure of HepG2 cells to bidentate chelators, *i.e.* MRB2 (A), MRB7 (B), MRB8 (C), and MRB9 (D), for 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 3 to 120 µM. Data are presented as mean ± standard error of the mean (SEM) of percentage of MTT reduction, relative to the negative controls (black bar), and were obtained in four independent experiments, performed in triplicates. The dotted red lines represent 50% and 100% effect. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *$p<0.05$, **$p<0.01$, and *** or **** $p<0.001$, when compared with control.

Considering the rate of MTT reduction, in general, results for bidentate (Figure 4) and hexadentate chelators (Figure 6) indicate that all fluorescent compounds concentration-dependently decrease the cell ability to reduce the MTT, although the chelators presented distinct cytotoxicity profiles. When bidentate chelators where tested at 70.5 µM, mitochondrial viability ranged from 88.34±4.37% (MRB2; $p>0.05$) to 59.46±12.90% (MRB7; $p<0.01$). A substantially pronounced deleterious effect was only consistently induced by all bidentate chelators at the concentration of 120 µM ($p<0.01$). Accordingly, this was the only concentration tested above the respective EC_{50} values (as denoted by responses below 50% effect, Figure 4). The exception was MRB9, which presented an EC_{50} value higher than 120 µM. At this concentration of ligand, 67.98±4.14% cells were viable, compared to controls; while other bidentate chelators, profoundly disturb cell reduction ability to levels as low as 27.71±15.63% (MRB7; $p<0.001$). Overall, MRB9 presented the lowest cytotoxic potency in the MTT assay (as determined by the calculated EC_{50} value), showing a milder toxicity profile when compared to the other chelators (Figure 5). Although at lower concentrations (<24.5 µM) the toxicity of MRB9 is slightly higher when compared to other test chelators, these differences in toxicity are rather small and are only seen at very low levels of cytotoxicity (<20% effect; Figure 5).

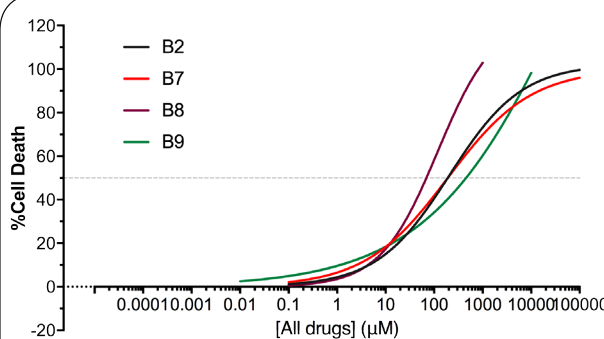

Figure 5. Regression models for the cell metabolic competence, as assessed by the reduction of the MTT assay, following exposure of HepG2 cells to bidentate chelators, *i.e.* MRB2 (black line), MRB7 (red line), MRB8 (purple line), and MRB9 (green line), for 5 days, at 37 °C. Data relative to the negative controls were from four independent experiments run in triplicate. The dotted line represent 50% effect.

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The analysis of the cytotoxic profiles obtained for hexadentate chelators by MTT assay evidenced that, in what relates to loss of metabolic competence, the two highest concentrations tested were already above the EC_{50} values for all chelators, *i.e.* from 23.5 µM up MTT reduction was below 50% of controls (Figure 6). Accordingly, MTT reduction at this concentration level ranged from 48.74±9.74% (MRH7; $p<0.001$) to 40.06±9.37% (MRH8; $p<0.01$). The MTT metabolism was further disrupted when hexadentate chelators were tested at 40 µM, with cell viability reaching values as low as 9.14±6.17% for MRH8 (Figure 3; $p<0.0001$), which was the chelator presenting the highest cytotoxic potency in the current assay.

Results of the MTT reduction assay in HepG2 cells in the presence of bidentate and hexadentate chelators are presented in Figures 4 and 6, respectively.

Discussion

Antimicrobial-resistant bacteria are a major concern of yet unpredictable proportions in public health. In this context, mycobacteria control urges the development of new drugs to circumvent the challenging mechanisms of resistance associated with poor therapeutic outcomes (Renvoise *et al.,* 2015). Aiming to provide attractive alternative therapies to control mycobacteria infection we synthesized a set of 3,4-HPO chelators and analysed their pharmacological bacteriostatic activity against *M. avium* (Fernandes *et al.,* 2010, Moniz *et al.,* 2013, Moniz *et al.,* 2015). Amongst the fluorescent chelators functionalized with different fluorophores, the rhodamine B isothiocyanate-labelled chelator MRH7 exhibited the strongest inhibitory activity of intramacrophagic *M. avium* growth (Fernandes *et al.,* 2010).

Following validation of the mycobacteriostatic activity of the new chelators, we further intended to appraise the *in vitro* safety of these bioactive derivatives, as this is a major requirement to pursue their therapeutic application. To achieve our purpose, HepG2 cells were selected, as they are vastly utilised as an *in vitro* model for the hepatocyte (Dykens *et al.,* 2008, Swiss & Will, 2011, Hynes *et al.,* 2013).

Because the liver is one of the main targets for drug toxicity, *in vitro* models representative of this organ are

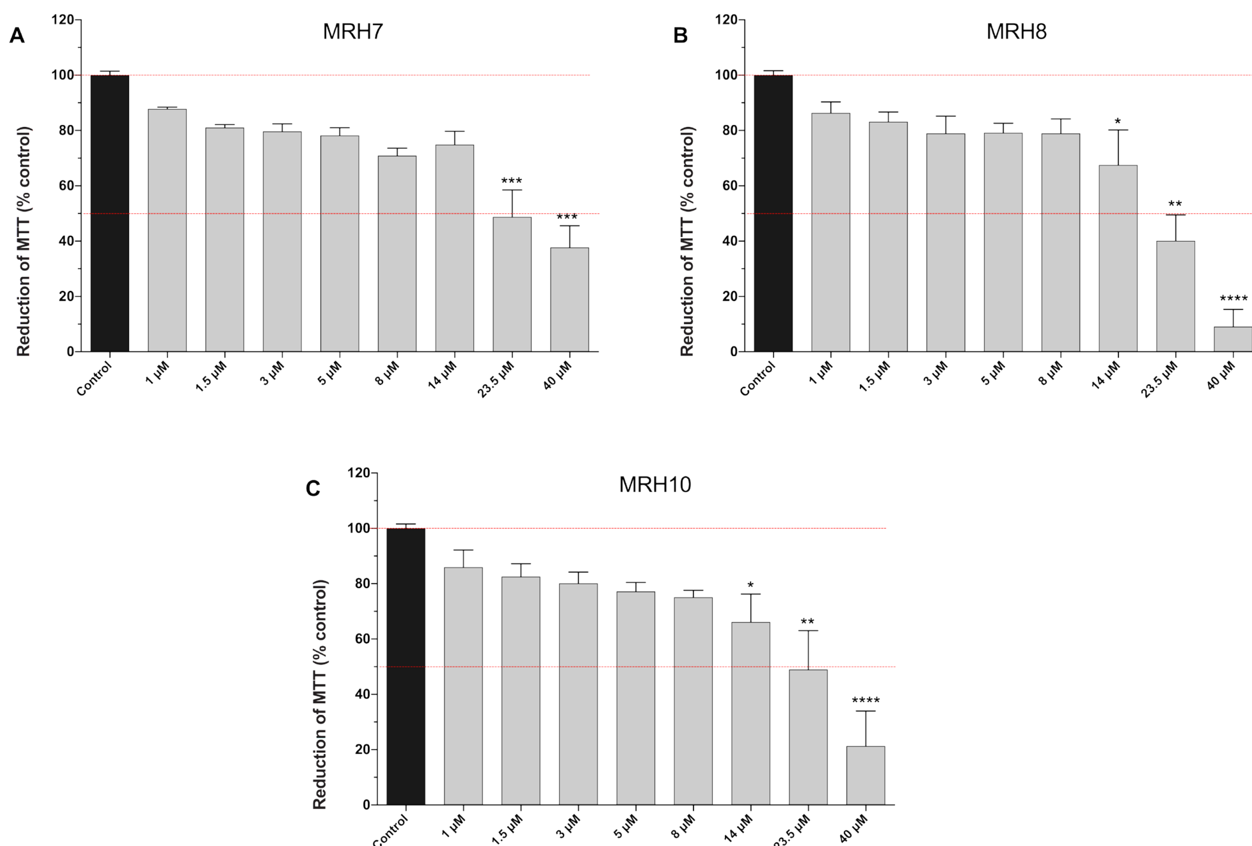

Figure 6. Cell metabolic competence, assessed by the reduction of the MTT assay, following exposure of HepG2 cells to hexadentate chelators, *i.e.* MRH7 (A), MRH8 (B), and MRH10 (C), for 5 days, at 37 °C. Each chelator was tested at concentrations ranging from 1 to 40 µM. Data are presented as mean ± standard error of the mean (SEM) of percentage of MTT reduction, relative to the negative controls, (black bar) and were obtained in four independent experiments, performed in triplicates. The dotted red lines represent 50% and 100% effect. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's multiple comparison *post hoc* test. *$p<0.05$, **$p<0.01$ and *** or **** $p<0.001$, when compared with control.

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of utmost suitability for the preliminary toxicological screening of new drugs. In this particular case, HepG2 cells preserve morphological and functional differentiation of hepatocytes *in vivo*; they are highly polarized, *i.e.* display cell asymmetry resultant of the presence of basolateral and apical poles and, in addition to the phenotypical features, they also express several enzymes involved in the metabolism and detoxification of xenobiotics, justifying the relevance of this model for the toxicological screening of our set of chelators (Sassa *et al.*, 1987, Knasmuller *et al.*, 2004, Mersch-Sundermann *et al.*, 2004, Decaens *et al.*, 2008, Gerets *et al.*, 2009, Moreira 2013).

Using HepG2 cells, the toxicity of the pharmacologically active rhodamine-labelled 3,4-HPO chelators was herein first investigated by the LDH assay, which is one of the most accepted tests for determination of toxicity of drugs *in vitro* (Weyermann *et al.*, 2005, Fotakis & Timbrell, 2006, Smith *et al.*, 2011, Brown *et al.*, 2014). According to LDH assay, the deleterious effect provoked by the chelators on cell membrane integrity was indirectly evaluated through the measurement of the extracellular LDH activity. The presence of this cytoplasmic enzyme in the extracellular medium is indicative of alterations on cell permeability; therefore, the higher the amount of enzyme released into extracellular culture medium, the higher the impairment of cell integrity triggered by chelators, which may directly result from the interaction of these compounds at the membrane, or may be a late event subsequent to the disruption of other cell organelles.

Despite the advantages of the LDH assay, such as its non-destructive nature, reliability, and quickness (Decker *et al.*, 1988, Smith *et al.*, 2011), it may also present some disadvantages. The enzyme has relatively short half-life in the medium what could introduce some artefacts on the obtained results (von Eyben *et al.*, 2001). Accordingly, an eventual degradation of the released enzyme may justify the low reproducibility observed at extended chelator exposures (5 day-incubations) for all chelators, regardless the concentration tested.

To overcome potential limitations of the LDH assay at the higher time point tested, a complementary assay was used to investigate the toxicity of the rhodamine labelled 3,4-HPO chelators. Accordingly, through the MTT assay, we measured the detrimental impact provoked at the metabolic level, after exposing HepG2 cells to bidentate and hexadentate chelators, for five days.

As cell metabolic activity is correlated with its viability, the cytotoxicity of the chelators was estimated considering the rate of MTT reduction (van Meerloo *et al.*, 2011).

Despite cytotoxicity tests such as the MTT assay being often used for the primary screening of new molecules (Berridge *et al.*, 2005, Fotakis & Timbrell, 2006), we have to consider some drawbacks. First, as the MTT assay is a destructive test (cells are lysed at the end of the experiment), hampered the possibility of evaluating the cytotoxicity at intermediate time points, along the time course of the experiment. Second, the formazans formed and the rhodamine-derived chelators have maximal absorbance in the same wavelength range (Nunes *et al.*, 2010, Moniz *et al.*, 2013, Moniz *et al.*, 2015). To preclude overestimation of the absorbance values recorded in the assay and, therefore, inaccurate conclusions of higher cell metabolic competence, controls were performed, in which absorbance readings occurred following aspiration of the treatment solutions – previous to addition of MTT solution, and no chelator-derived pigmentation was detected (purple coloration only occurred after MTT metabolism).

Regarding the conditions of our testing system, another aspect should be considered. It is well established that long incubation periods largely impact cell viability. Results obtained in MTT assay were achieved after 5 day-chelator exposures, this way allowing comparisons with the studies conducted to evaluate the antimycobacterial effects of the chelators (Moniz *et al.*, 2015). During that period, the cell culture medium was not changed and cells were not split to provide room for growth. Therefore, some level of basal stress naturally occurred, which could potentiate the toxicity induced by chelators.

From the above mentioned, it is important to highlight that the toxicity observed for chelators after 5-day exposures in both LDH and MTT assays is very likely overestimated but this provides more cautious estimations of the deleterious effects elicited by these chelators. In addition, compared to LDH assay, MTT method measures an earlier toxicological phenomenon (under most conditions, metabolic damage occurs prior to cytolysis), contributing to more conservative assumptions. Therefore, conclusions from the current study will mostly rely on the results attained in the MTT test.

Taken together, the results obtained herein shed light on the relationship toxicity-structure of the rhodamine-labelled 3,4-HPO chelators, unveiling that bidentate ligands are less detrimental to cells than hexadentate chelators, yet the test concentrations were 3x higher (*e.g.* MRB7 and MRB8 are less toxic than MRH7 and MRH8, respectively). Notwithstanding, hexadentate chelators revealed to be the most effective chelators against *M. avium* (Moniz *et al.*, 2013, Moniz *et al.*, 2015), indicating a strict correlation between this pharmacological activity and toxicity, which may be directly dependent on the size of the molecule, and/or on the hydrophilic/lipophilic balance (Moniz *et al.*, 2017). These properties may influence the ability of the chelators to interact and/or cross biological membranes and ultimately to reach their targets, namely the phagosome where *M. avium* resides. Similarly, the capacity of the chelators to interact with biological membranes may also be related to their toxicity, as it affects the availability of the drug at the toxicological targets, such as mitochondria, lysosome or endoplasmic reticulum.

Confocal microscopy studies have shown that endocytosis is not the preferred pathway for the cellular uptake of 3,4-HPO chelators (Moniz *et al.*, 2017). Therefore, the permeation thought the biological membranes must be determinant for their final toxicological effects. Previous experiments on the interaction of the chelators with membranes revealed that the compounds that herein presented higher toxicity strongly interact with the lipid

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phase, rapidly permeating into the cell, and further reaching cytosol and phagosome. Accordingly, the intracellular uptake of rhodamine B isothiocyanate derivatives MRH7 and MRB7 was more efficient than that observed for the carboxytetramethylrhodamine chelator MRH8, which is in line with the observation that the interaction with the hydrophobic region of the membrane is strengthened by the presence of the *N*-ethyl groups and the thiourea linkage (Coimbra *et al.,* 2014, Moniz *et al.,* 2016b).

Also, in what concerns to the chemical structure, our data signpost the relevance of the linker and of the substituents of nitrogen atom on the toxicity exhibited by chelators. Among the set of bidentate ligands, carboxyrhodamine derivatives (amide linker), like MRB8 and MRB9, present lower detrimental effects, when compared with rhodamine B isothiocyanate chelators (thiourea linker; MRB7). A similar relationship structure–antibacterial activity against *M. avium* was also established (Moniz *et al.,* 2013, Moniz *et al.,* 2015). Contrarily, for the hexadentate series, rhodamine B isothiocyanate derivatives (MRH7 and MRH10) are less cytotoxic than the carboxyrhodamine molecules (MRH8), but more active against *M. avium* (Moniz *et al.,* 2013, Moniz *et al.,* 2015) and other Gram positive and Gram negative bacteria (Moniz *et al.,* 2018). For all chelators tested (hexadentate and bidentate series), when the substituents of the nitrogen atom are switched from ethyl to methyl (MRH7 compared to MRH10 and MRB9 compared to MRB8), an increment of toxicity was observed.

Of note, MRB2 chelator is an exception to the former structure–bacteriostatic activity deductions; as this is the only molecule displaying both a sulphonamide liker and a sulfonate group on the position 2’ of the phenyl ring, comparisons to other derivatives are precluded as differences to toxicological profiles cannot be unequivocally attributed to the presence of the linker or of the SO_3^- group. Notwithstanding, these structural properties might be responsible for the absence of bacteriostatic activity against *M. avium* and for the low toxicity displayed by the chelator.

Overall, our results indicate that an incubation time of five days might be excessively long for the majority of the testing chelators, when tested at the highest concentrations. A feasible treatment approach to fight infection of *M. avium* would be using lower concentrations of the most active chelators, such as 5–14 μM MRH7 or 5–8 μM MRH8, since at this concentration ranges pharmacological activity is already observed with no significant toxicological impact (Moniz *et al.,* 2015). Contrarily, MRB9 was the substance tested that revealed the worst benefit: risk ratio, as it only disclosed an antimycobacterial tendency at concentrations as high as 60 μM, but presented significant toxicity from 8.5 μM on (Moniz *et al.,* 2015). Other chelators, such as MRB7, MRH8, and MRB8 also displayed significant inhibition of intramacrophagic *M. avium* growth at very low concentrations but deductions on potential benefit: risk are precluded by the different experimental settings (*i.e.* incubation time) of the pharmacological and toxicological assessments (Moniz *et al.,* 2013). Furthermore, as our chelators presented no cytotoxicity at 1 day-incubation, their applicability as antibiotics may be also relevant to fight fast growth mycobacteria (*e.g.*, *M. smegmatis*) or others requiring short term antibiotic regimens. Accordingly, MRH7 already demonstrated significant antimicrobial activity against *Staphylococcus aureus, S. epidermis*, and *Escherichia coli*, after 1 day (Gomes *et al.,* 1999, Cronje & Bornman, 2005). In addition, MRH10, MRH8, MRB9, MRB8 and MRB7 were also active against *S. aureus*; and MRH10, MRB9, MRH8, and MRB8 against *S. epidermis* (Moniz *et al.,* 2018).

Overall, the integration of antimycobacterial properties with the toxicity profiles enabled ranking chelators according to their pharmacological: toxicological potential ratio and identification of MRH7 and MRH8 as the most suitable chelators to undergo further pre-clinical development studies.

Conclusion

Our results show that only after long periods of incubation, *i.e.* 5 days, the rhodamine-labelled chelators induce significant toxicity to HepG2 cells; for earlier time-points, the cytotoxicity was inexistent or low, supporting a potential clinical applicability of all chelators tested herein as bacteriostatic agents against fast growth bacteria. Bidentate chelators were the least toxic chelators, in particular MRB2, MRB7, and MRB9. Amongst the hexadentate series, MRH7, which is the most active chelator against *M. avium*, was the least toxic agent; at lower concentrations, MRH8 might also be a reasonable strategy to treat *M. avium*.

To the best of our knowledge, this work is the first report concerning the *in vitro* cytotoxic profile of these chelators, which gains particular relevance since these chelators display high potential to be clinically applied as antibacterial agents. In this regard, these present results improved our knowledge on the contribution of the different chemical groups in the molecular framework for the toxicity of the molecule, a key point to be considered in the molecular design of novel chelators.

Further investigations aiming to thoroughly elucidate the mechanisms underlying the toxicity we described herein and establish the metabolic profile of these chelators will be of utmost relevance, particularly in the case of MRH7 and MRH8.

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REFERENCES

al-Khafaji B, Kralovic S, Smith RD. (1997). Increased hepatic iron in the acquired immunodeficiency syndrome: an autopsy study. *Mod Pathol* **10**: 474–480.

Amit A, Chaudhary R, Yadav A, Suman SS, Narayan S, Das VN, Pandey K, Singh SK, Singh BK, Ali V, Das P, Bimal S. (2014). Evaluation of Leishmania donovani disulfide isomerase as a potential target of cellular immunity against visceral leishmaniasis. *Cell Immunol* **289**: 76–85.

Berridge MV, Herst PM, Tan AS. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev* **11**: 127–152.

Brown DM, Johnston H, Gubbins E, Stone V. (2014). Serum enhanced cytokine responses of macrophages to silica and iron oxide particles and nanomaterials: a comparison of serum to lung lining fluid and albumin dispersions. *J Appl Toxicol* **34**: 1177–1187.

Coimbra JT, Moniz T, Brás NF, Ivanova G, Fernandes PA, Ramos MJ, Rangel M. (2014). Relevant interactions of antimicrobial iron chelators and membrane models revealed by nuclear magnetic resonance and molecular dynamics simulations. *J Phys Chem B* **118**: 14590–14601.

Cronje L, Bornman L. (2005). Iron overload and tuberculosis: a case for iron chelation therapy. *Int J Tuberc Lung Dis* **9**: 2–9.

da Silva DD, Silva E, Carmo H. (2014). Combination effects of amphetamines under hyperthermia – the role played by oxidative stress. *J Appl Toxicol* **34**: 637–650.

Decaens C, Durand M, Grosse B, Cassio D. (2008). Which in vitro models could be best used to study hepatocyte polarity? *Biol Cell* **100**: 387–398.

Decker T, Lohmann-Matthes ML. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* **115**: 61–69.

Dias da Silva D, Carmo H, Silva E. (2013). The risky cocktail: what combination effects can we expect between ecstasy and other amphetamines? *Arch Toxicol* **87**: 111–122.

Dykens JA, Jamieson JD, Marroquin LD, Nadanaciva S, Xu JJ, Dunn MC, Smith AR, Will Y. (2008). In vitro assessment of mitochondrial dysfunction and cytotoxicity of nefazodone, trazodone, and buspirone. *Toxicol Sci* **103**: 335–345.

Fernandes SS1, Nunes A, Gomes AR, de Castro B, Hider RC, Rangel M, Appelberg R, Gomes MS. (2010). Identification of a new hexadentate iron chelator capable of restricting the intramacrophagic growth of Mycobacterium avium. *Microbes Infect* **12**: 287–294.

Fotakis G, Timbrell JA. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* **160**: 171–177.

Gerets HH, Hanon E, Cornet M, Dhalluin S, Depelchin O, Canning M, Atienzar FA. (2009). Selection of cytotoxicity markers for the screening of new chemical entities in a pharmaceutical context: a preliminary study using a multiplexing approach. *Toxicol in Vitro* **23**: 319–332.

Gomes MS, Dom G, Pedrosa J, Boelaert JR, Appelberg R. (1999). Effects of iron deprivation on Mycobacterium avium growth. *Tuber Lung Dis* **79**: 321–328.

Hynes J, Nadanaciva S, Swiss R, Carey C, Kirwan S, Will Y. (2013). A high-throughput dual parameter assay for assessing drug-induced mitochondrial dysfunction provides additional predictivity over two established mitochondrial toxicity assays. *Toxicol in Vitro* **27**: 560–569.

Knasmüller S, Mersch-Sundermann V, Kevekordes S, Darroudi F, Huber WW, Hoelzl C, Bichler J, Majer BJ. (2004). Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. *Toxicology* **198**: 315–328.

Mersch-Sundermann V, Knasmüller S, Wu XJ, Darroudi F, Kassie F. (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology* **198**: 329–340.

Moniz T, Coimbra JTS, Brás NF, Cunha-Silva L, Ramos MJ, Fernandes PA, de Castrob B, Rangel M. (2016a). Synthesis and structural characterization, by spectroscopic and computational methods, of two fluorescent 3-hydroxy-4-pyridinone chelators bearing sulphorhodamine B and naphthalene. *RSC Advances* **6**: 4200–4211.

Moniz T, de Castro B, Rangel M, Ivanova G. (2016b). NMR study of the interaction of fluorescent 3-hydroxy-4-pyridinone chelators with DMPC liposomes. *Phys Chem Chem Phys* **18**: 5027–5033.

Moniz T, Feio M, Silva D, de Castro B, Range M. (2018). Study of the effect of thiourea and N-ethyl groups on antibacterial activity of rhodamine-labelled 3,4-HPO iron Chelators against Gram (+/–) bacteria. *Med Chem Res* **27**: 1472–1477.

Moniz T, Leite A, Silva T, Gameiro P, Gomes MS, de Castro B, Rangel M. (2017). The influence of functional groups on the permeation and distribution of antimycobacterial rhodamine chelators. *J Inorg Biochem* **175**: 138–147.

Moniz T, Nunes A, Silva AM, Queirós C, Ivanova G, Gomes MS, Rangel M. (2013). Rhodamine labeling of 3-hydroxy-4-pyridinone iron chelators is an important contribution to target Mycobacterium avium infection. *J Inorg Biochem* **121**: 156–166.

Moniz T, Silva D, Silva T, Salomé Gomes M, Rangel M. (2015). Antimycobacterial activity of rhodamine 3,4-HPO iron chelators against Mycobacterium avium: analysis of the contribution of functional groups and of chelator´s combination with ethambutol. *MedChemComm* **6**: 2194–2203.

Moreira AC. (2013). Phytoestrogens as Alternative to the Hormone Replacement Therapy: Mitochondrial and Cellular Interactions. PhD thesis in Bio-Sciences, Faculty of Sciences and Technology, University of Coimbra.

Nunes A, Podinovskaia M, Leite A, Gameiro P, Zhou T, Ma Y, Kong X, Schaible UE, Hider RC, Rangel M. (2010). Fluorescent 3-hydroxy-4-pyridinone hexadentate iron chelators: intracellular distribution and the relevance to antimycobacterial properties. *J Biol Inorg Chem* **15**: 861–877.

Rajapakse N, Silva E, Scholze M, Kortenkamp A. (2004). Deviation from Additivity with Estrogenic Mixtures Containing 4-Nonylphenol and 4-tert-Octylphenol Detected in the E-SCREEN Assay. *Environ Sci Technol* **38**: 6343–6352.

Renvoisé A, Brossier F, Galati E, Veziris N, Sougakoff W, Aubry A, Robert J, Cambau E, Jarlier V, Bernard C. (2015). Assessing primary and secondary resistance to clarithromycin and amikacin in infections due to Mycobacterium avium complex. *Antimicrob Agents Chemother* **59**: 7153–7155.

Sassa S, Sugita O, Galbraith RA, Kappas A. (1987). Drug metabolism by the human hepatoma cell, Hep G2. *Biochem Biophys Res Commun* **143**: 52–57.

Silva-Gomes S, Vale-Costa S, Appelberg R, Gomes MS. (2013). Iron in intracellular infection: to provide or to deprive? *Front Cell Infect Microbiol* **3**: Article 96, 91–11.

Smith SM, Wunder MB, Norris DA, Shellman YG.. (2011). A simple protocol for using a LDH-based cytotoxicity assay to assess the effects of death and growth inhibition at the same time. *PLoS One* **6**: e26908.

Swiss R, Will Y. (2011). Assessment of mitochondrial toxicity in HepG2 cells cultured in high-glucose- or galactose-containing media. *Curr Protoc Toxicol.* John Wiley & Sons, Inc., Chapter 2, Unit2.20.

Thompson KH, Barta CA, Orvig C. (2006). Metal complexes of maltol and close analogues in medicinal inorganic chemistry. *Chem Soc Rev* **35**: 545–556.

van Meerloo J, Kaspers GJ, Cloos J. (2011). Cell sensitivity assays: the MTT assay. *Methods Mol Biol* **731**: 237–245.

von Eyben FE, Madsen EL, Blaabjerg O, Petersen PH, von der Maase H, Jacobsen GK, Rørth M. (2001). Serum lactate dehydrogenase isoenzyme 1 and relapse in patients with nonseminomatous testicular germ cell tumors clinical stage I. *Acta Oncol* **40**: 536–540.

Weyermann J, Lochmann D, Zimmer A. (2005). A practical note on the use of cytotoxicity assays. *Int J Pharm* **288**: 369–376.

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ORIGINAL ARTICLE

Effects of mustard oil cake on liver proteins of *Channa punctatus* (Bloch)

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ABSTRACT
Mustard oil cake is a biofertilizer widely used in agriculture and fish cultivation almost in all South East Asian Countries including India. The study was carried out to observe the effects of this biofertilizer on the liver proteins of *Channa punctatus*. At sublethal concentration (0.42 g/L), fishes were exposed for a prolonged period of 35 days and amount of total liver protein (TLP) was measured. The investigation showed a low rate of liver protein synthesis in treated fish after 4 days of exposure. An increase in the amount of protein was observed between the 7th and 35th day. But such increment was below the amount of TLP of control fish, indicating physiological stress in the treated fish.

KEY WORDS: mustard oil cake; *Channa punctutas*; liver; protein

Introduction

Inorganic fertilizer is used to increase the growth and production of cultured plant and fish. But it has some adverse effect on the environment. Nowadays organic fertilizer as well as biofertilizer are used to reduce the adverse effects on the environment and induce growth performance, as reported by Abbas *et al.* (2001) on the major carp. Investigation showed a change in carbohydrate and nitrogen metabolism, depletion of protein glycogen and pyruvate stored in liver and muscle of fish during stress by pesticide induced hypoxia (Laul *et al.*, 1974). Mukhapadhyay and Dehadrai (1987) investigated the metabolic fate of non-protein nitrogenous substance for urea in *Heteropnustus fossilis.* The capacity of fish to survive on decayed or detritus matter and the capacity of tolerance of high concentration of ambient ammonia was revealed by Dehadrai (1980). Vasait and Patil (2005) studied the effect of monocrotophos on edible fish and observed a marked reduction in hemoglobin and total erythrocyte count. The study also revealed changes in liver and muscle protein concentration of *Channa punctatus* depending upon the period of exposure and concentration of xenobiotics applied (Sirohi & Saxena, 2006). Naveed *et al.* (2010) reported reduction in the level of total proteins and significant enhancement of free amino acid when *Channa punctatus* was exposed to triazophos. Malathi *et al.* (2012) studied the comparative hematological parameter on *Channa punctatus* in reference to physiological stress. Ahmed (2013) studied the effect of industrial waste discharge on the physiological parameter of *Tilapia niloticus*. Maitra and Nath (2014) studied the impact of urea on the hematological parameter of *Heteropnustus fossilis* and revealed the recovery pattern from the negative effect of toxic material. This toxic chemical after reaching sufficiently high concentration in body cell may cause alteration in physiological function of the aquatic organisms (Heath, 1987; Bartoskova *et al.*, 2013; Torre *et al.*, 2013; Fazio *et al.*, 2014; Aliko *et al.*, 2015; Faggio *et al.*, 2016; Pagano *et al.*, 2016; Pagano *et al.*, 2017; Savorelli *et al.* 2017). Khan Niazi (1986) reported that mustard oil cake contained a high amount of the protein allylisothiocyanate, phytic acid, *etc.* Mondal *et al.* (2014) reported that the level of accumulation of mineral, nitrogen in soil was much more pronounced when mustard oil cake was applied along with other edible and non-edible oil cake.

So far there is no such study which can explore the effects of mustard oil cake on physiological aspects of liver protein of fish. Here an attempt has been made to observe the effect of mustard oil cake on *Channa punctatus* to liver protein levels during various days of exposure.

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Material and methods

Selection of specimen

Air breathing teleost, *Channa punctatus* (Bloch), commonly known as snake headed fish, were collected from the market of Dumdum, Kolkata, West Bengal. Adult fish of average weight (51.68±0.634), were selected for the experiment. Infected and diseased fish were avoided.

Rearing and culture

Live fresh water fish, *Channa punctatus*, was collected and cleaned with 0.2% $KMnO_4$ solution to avoid any fungal infection. Then the fishes were stored in a glass aquarium (90 cm × 50 cm × 31 cm) containing tap water and were acclimated under laboratory condition for 7 days. Water was changed every 48 hr. Dead fish (if any) were removed as soon as possible. Commercial fish food (containing crude protein 46%, fat 6%, fiber 5%) was provided during acclimatization as well as treatment period at the rate of only 2% of the fish body weight, once a day. The laboratory photo period was 12 hr. dark: 12 hr. light.

Biofertilizer

Bio-fertilizer, namely Mustard Oil cake (MOC), was used in the experiment. It is widely used in major cultivation in West Bengal, such as paddy, wheat, potato, *etc.* Dry MOC is mixed with the soil by ploughing to form seed bed. The measurement of applied MOC during cultivation is 50 kg/acre. Run off from the field in nearby water body may affect fishes and other animals at high concentration.

The chemical composition of MOC is: 43% protein, 2.05% oil, 1.22% Allylisothiocyanate (AITC) and 2.75% phytic acid. Phytic acid is usually regarded as an antinutritive factor (Khan Niazi, 1986).

Treatment

Acclimatized fishes were treated with MOC, a biofertilizer widely used in agriculture. LC_{50} was measured as 0.4625 g/L (96 hr.) during this experiment, according to the probit analysis method (Finney 1971). Acclimatized fishes were exposed (EP) to sub-lethal concentration (0.42 g/L) of MOC based on the result of 96 hr. LC_{50}. One aquarium (90 cm × 50 cm × 31 cm) was set for that particular dose and 30 fishes were kept in 60 L of tap water. The water temperature was kept at 28±1 °C during the whole experimental period. Another aquarium containing the same number of fish was maintained as control for the experiment. Water quality was maintained approximately as pH 7, alkalinity=293 mg/L, hardness=388 mg/L, nitrate=0.85 and DO=10.02 mg/L during the experiment. During the treatment period five fish were sacrificed at a time after 4 days, 7 days, 14 days, 21 days, 28 days and 35 days. The liver was isolated for protein estimation from five fish separately for each day of exposure. No fish were found to die either in the control or treated groups.

Protein concentration

Liver tissues were collected from the specimen and wet weight was measured. Then it was homogenized in a glass homogenizer (REMI Cat.No.RQ127A) using 10 mL of phosphate buffer solution (0.1 M, pH 7.4) as suggested by Saito *et al*. (1983) and centrifuged by cooling centrifuge (REMI serial No. EVCI 6169). Protein level was assessed according to the method of Lowry *et al.* (1951) using visual Spectrophotometer (SYSTRONICS 117). OD values were obtained and the protein concentration was calculated against bovine serum albumin as standard. Values have been expressed as mg/g.

Liver somatic index

Liver somatic Index (liver weight as % of body weight) was calculated with respect to the total body weight (Heidinger & Crawford, 1977).

Statistical analysis

ANOVA (single factor) was done by using Origin 6.0. Strength of association (ω^2) were also calculated with the recorded data (Das & Das, 1993).

Results

Channa punctatus exposed to water treated with 0.42 g/L of Mustard Oil Cake showed no mortality after prolonged period of exposure but the fishes were at physiological stress (Natarajan, 1984). In that condition the Total Liver Proteins (TLP) were estimated. In non-treated fish the level of TLP ranged between 85.8±0.59 mg/g and 128.4±0.464 mg/g and indicated a steady rate of increase with the advancement of days (Table 1). But there was a significant ($p<0.05$) decrease of protein content in the liver during various days of exposure. The rate of decrease in TLP was rapid on the 4th day, then an increase in the level of proteins was observed though the amount was lower than in the control fish as also revealed in the Liver Somatic Index (Table 2). Strength of Association (ω^2) is made to estimate the degree of relatedness between duration of exposure and liver protein concentration. The computed values show the proportion as 0.99(EP=35) >0.95(EP=07) >0.94(EP=28) >0.82(EP=21) >0.47(EP=4) >0.38(EP=14) of total variance of protein concentration as related to the duration of the exposure (Table 3).

Discussion

The result of the experiment revealed that protein concentration of the liver after 4 days of MOC treatment was significantly lower than the control. Decrease in the amount of liver proteins during the first 4 days of treatment showed that the fish were at physiological stress. According to Lett *et al.* (1976) the reduction in protein level may lead to increase in energy demand at the time of stress. As an important constituent of all the cells and tissue, proteins play an important role in physiological activity of living organisms (Adamu & Saikpere, 2011; Burgos-Aceves *et al.*, 2016; Lauriano *et al.* 2016). Moreover, proteins act as a source of energy during the

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Table 1. Amount of liver protein mg/g in treated and non-treated *Channa punctatus*.

Type of specimen	Day 0	Day 4	Day 7	Day 14	Day 21	Day 28	Day 35
Control	85.8±0.59	65.6±2.16	110.72±0.28	109.04±0.66	108.64±0.24	127.34±0.57	128.4±0.464
Treated	–	54.5±0.94	94.01±0.73	103.15±1.36	94.51±1.35	107.93±0.92	85.4±0.38

Liver Protein as mg/g (mean±SE)

Table 2. Liver somatic index

Control	Day 4	Day 7	Day 14	Day 21	Day 28	Day 35
0.8126%	0.935%	1.21%	1.14%	1.10%	1.11%	0.88%

Table 3. Statistical relations between control and treated *Channa punctatus*

Statistics	Day4	Day7	Day14	Day21	Day28	Day 35
F*	19.87	399.59	13.64	96.17	309.01	521.39
ω^2	0.47	0.95	0.38	0.82	0.94	0.99
t-test†	4.45	19.98	3.69	9.8	17.57	72.21
Bonferroni modification	*p*<0.002	*p*<0.002	*p*<0.002	*p*<0.002	*p*<0.002	*p*<0.002

*Significant *p*<0.05; † Significant *p*<0.001

chronic period of stress (Umminger, 1977). But with advancement of days, there was rapid increase in protein concentration at first, then the rate of increase maintained a steady state which was higher than on 4th day as well as in control. This might indicate fish perseverance to cope with adverse stress situation. Moreover, TLP remained at low level in all treated fish compared to untreated. So more proteins were used to meet the increased energy demand, which led to increase the rate of protein synthesis. The used culture medium contaminated with MOC contains 43% protein (Khan Niazi, 1986) and it is also a rich source of nitrogen (Mondal *et al.*, 2014). Abbas *et al.* (2001) reported an average weight gain of fish when the pond was treated with urea. Whereas Tarar (1997) obtained higher net fish production from a pond which was urea treated as a source of non-protein nitrogen and a better nitrogen incorporation efficiency. MOC contains 43% protein. Based on the present experiment it was probable that increase in protein synthesis was accelerated by the consumption of protein and nitrogen from the culture medium by the fish. Increase in the synthesis of liver protein was probably due to metabolism of proteins synthesis enzyme activities in the fish and MOC stimulate the rate of synthesis during prolonged exposure. Adamu and Siakpere (2011) proposed that protein is the chief source of nitrogen metabolism. During the long-term exposure, protein concentration shows gradual increase. The rate of protein synthesis or its degradation regulates the quantity of protein. Moreover , impaired incorporation of amino acids in the polypeptide chain also affect the quantity of protein (Singh *et al.* 1996). On the other hand, inhibition of alkaline phosphatase activity reduces the protein level, as it plays an important role in protein synthesis along with the other secretory activities (Pilo *et al.*, 1972; Ibrahim *et al.*, 1974). The outcome of the study was that MOC alone was not sufficient for TLP synthesis, as artificial fish food which was provided at the rate of 2% of body weight, a very negligible amount as the body weight of experimental fish was concerned.

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REFERENCES

Abbas S, Ahimed L, Akhter P, Asghar T. (2001). Response of urea on the growth performance of major carp viz.,*Labeo rohita, Catla catla* and *Chirinus mrigala. Pakistan Vet J* **21**(4): 180–183.

Adamu KM, Siakpere OK. (2011). Effect of sub lethal concentration of tobacco(*Nicotina tobaccum)*leaf dust on some biochemical parameters of Hybrid Catfish(*Clarias gariepinus* and *Heterobranchus bidorsalis. Braz Arch Biol Technol* **54**(1): 183–196.

Ahmed DFI. (2013). Effect of industrial waste discharge including heavy metals Burullus lake on some physiological parameter and antioxidants in *Tilapia niloticus* and *Siluriforms* fish. *N Y Sci J* **6**(4): 85–92.

Aliko V, Hajdaraj G, Caci A, Faggio C. (2015). Copper Induced Lysosomal Membrane Destabilisation in Haemolymph Cells of Mediterranean Green Crab (*Carcinus aestuarii*, Nardo, 1847) from the Narta Lagoon (Albania). *Braz. Arch. Biol. Technol* **58**(5): 750–756.

Bartoskova M, Dobsikova R, Stancova V, Zivna D, Blahova J, Marsalek P, Zelníckova L, Bartos M, Di Tocco FC, Faggio C. (2013). Evaluation of ibuprofen toxicity for zebrafish (*Danio rerio*) targeting on selected biomarkers of oxidative stress. *Neuro Endocrinol Lett* **34**: 102–108.

Bereitgestellt von University Library Bratislava | Heruntergeladen 07.02.20 13:14 UTC

Burgos-Aceves MA, Cohen A, Smith Y, Faggio C. (2016). Estrogen regulation of gene expression in the teleost fish immune system. *Fish Shellfish Immunol* **58**: 42–49.

Das D, Das A. (1993). *Statistics in Biology and Psychology*,2nd Edn. Academic Publishers, Calcutta, India, p.208.

Dehadrai PV. (1980). Vth Workshop Report of All India Co-ordinated Research Project on Air-breathing fish culture, pp.31–32.

Fazio F, Piccione G, Tribulato K, Ferrantelli V, Giangrosso G, Arfuso F, Faggio C. (2014). Bioaccumulation of Heavy Metals in Blood and Tissue of Striped Mullet in Two Italian Lakes. *J Aquat Anim Health* **26**: 278–284.

Faggio C, Pagano M, Alampi R, Vazzana I, Felice MR. (2016). Cytotoxicity, haemolymphatic parameters, and oxidative stress following exposure to sublethal concentrations of quaternium-15 in *Mytilus galloprovincialis*. *Aquat Toxicol* **180**: 258–265.

Finney DJ. (1971). *Probit Analysis*, Third Edition, Cambridge University Press, London,p: 333.

Heath AG. (1987). *Water Pollution and fish Physiology*, CRC Press, Boca Raton, Florida, p: 245.

Heidinger CR & Crawford SD.(1977). Effect of Temperature and Feeding rate on Liver-Somatic Index of the Largemouth Bass, *Micropterus salmoides*. *J Fish Res Board Can* **34**(5): 633–638.

Ibrahim AM, Higazi MG , Demian S.(1974). Histochemical localization of alkaline phosphatase activity in alimentary tract of snail *Marisa cornuarietes* (L). *Bull Zool Soc Egypt* **26**: 94–105.

Lauriano ER, Pergolizzi S, Capillo G, Kuciel M, Alesci A, Faggio C. (2016). Immunohistochemical characterization of Toll-like receptor 2 in gut epithelial cells and macrophages of goldfish *Carassius auratus* fed with a high-cholesterol diet. *Fish Shellfish Immunol* **59**: 250–255.

Khan Niazi AH. (1986). *Improvement in the Nutritive Value of Mustard seed Cake*. Doctoral thesis, University of Punjab, Lahore, Pakistan. pp: 13–14.

Laul RT, Pradhan PV, Bhagwat AM. (1974). Effect of muscular exercise on glycogen content in *Tilapia mossambica* (in captivity). *J Biol Chem* **17**: 72–77.

Lett PF, Farmer GJ, Beamish FWH. (1976). Effect of copper on some aspect of Bioenergetics of Rainbow trout(*Salmo gairdeneri*). *J Fish Res Board Can* **33**: 1335–1342.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. (1951). Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–257.

Maitra S, Nath S. (2014). Toxic impact of urea on the *Heteropnustus fossilis* (Bloch). *American-Eurasian J Agric & Environ sci* **14**(4): 336–334.

Malathi K, Kannathasan AK, Rejendran K. (2012).Comparetive haematological studies on Fresh water fishes *Channa punctatus* and *Channa striatus* (Bloch). *Int J Pharm Chem Biol Sci* **2**(4): 644–648.

Mondal, S, Das R, Das AC. (2014). A comparative study on the decomposition of edible and non-edible oil cake in Gangetic alluvial soil of West Bengal. *Environ Monit Assess* **186**(8): 5199–5207

Mukhopadhyay PK & Dehadrai PV. (1987). Metabolic rate of dietary non-protein nitrogen in *Heteropnustus fossilis* (Bloch). *Ind J Fisheries* **34**(3): 237–244.

Nataranjan GM. (1984). Effect of sub-lethal concentration of matasystox on selective oxidative enzyme,tissue respiration and haematology of the fresh water air-breathing fish, *Channa striatus*. *Pestic Biochem Physiol* **21**(2): 194–198.

Naveed A, Venkateshwarlu P, Janaiah C. (2010) Impact of sub lethal concentration of Triazophos on regulation of protein metabolism in the fish *Channa punctatus(*Bloch). *Afr J Biotechnol* **9**(45): 7753–7758.

Pagano M, Capillo G, Sanfilippo M, Palato S, Trischitta F, Manganaro A, Faggio C. (2016). Evaluation of Functionality and Biological Responses of *Mytilus galloprovincialis* after Exposure to Quaternium-15 (Methenamine 3-Chloroallylochloride). *Molecules* **21**(2): 144.

Pagano M, Porcino C, Briglia M, Fiorino E, Vazzana M, Silvestro S, Faggio C. (2017). The influence of exposure of cadmium chloride and zinc chloride on haemolymph and digestive gland cells from *Mytilus galloprovincialis*. *Int. J. Environ. Res* **11**(2): 207–216.

Pilo B, Asnani MV, Shah RV. (1972) Studied on wound healing and repair in pigeon III. Histochemical studies on acid alkaline phosphatase activity during the process. *J Ani Mor Phy* **19**: 205–212.

Saito T, Iso N, Mizuno H, Ohzeki F., Suzuki A, Kato T, Sekikawa Y.(1983). Effect of thermal treatment on extraction of protein from meats. *Bull Jpn Soc Sci Fish* **49**(10): 1569–1572.

Savorelli F, Manfra L, Croppo M, Tornambè A, Palazzi D, Canepa S, Trentini PL, Cicero AM, Faggio C. (2017). Fitness Evaluation of *Ruditapes philippinarum* Exposed to Ni. *Biol Trace Elem Res*. **177**(2): 384–393.

Singh A, Singh DK, Mishra, TN, Agarwal RA. (1996). Molluscicides of plant origin. *Bio Agric Horti* **13** : 205–252.

Sirohi V, Saxena KK. (2006).Toxic effect of λ-Cyhalothrin on biochemical contacts of Fresh water fish *Channa punctatus*. *J Fisheries Aquatic Sci* **1**(2): 112–116.

Tarar SR. (1997).*Comparison of fish length and weight based doses of inorganic fertilizer in pond fisheries.* M.Sc. Thesis, Department of Zoology and Fisharies *Univ of Agri* Faisalabad. P.78.

Torre A, Trischitta F, Faggio C. (2013). Effect of CdCl2 on Regulatory Volume Decrease (RVD) in *Mytilus galloprovincialis* digestive cells. *Toxicology in Vitro* **27**: 1260–1266.

Umminger BL. (1977). Relation of whole blood sugar concentration in vertebrate to standard metabolic rate. *Comp Biochem Phys. A* **55**: 457–460.

Vasait JD, Patil VT. (2005). The toxic evaluation of organophosphorus insecticide monocrotophos on the edible fish species *Nemacheilus botia. Eco Environ & Conservation* **8**(1): 95–98.

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ORIGINAL ARTICLE

New biological findings of ethanol and chloroform extracts of fungi *Suillellus rubrosanguineus* and *Tylopilus felleus*

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ABSTRACT

The aim of the research was to determine some basic biological activities of less biomedically studied but commonly known two fungi from the Boletaceae family *Suillellus rubrosanguineus* and *Tylopilus felleus*, which grow in the forests of Middle Europe. The cytotoxicity tests of the ethanol and chloroform extracts were carried out using NIH-3T3 and MCF-7 cell lines. The presence of alkaloids in the extracts was assessed by the reaction with Dragendorff reagent. In all of the extracts the positive reaction with the reagent was observed. In general, the extracts from *Suillellus rubrosanguineus* were more cytotoxic than the extracts from *Tylopilus felleus* and exhibited no selectivity of activities on healthy and cancer cell lines. However, the extracts from *Tylopilus felleus* proved to be selectively cytotoxic for cancer cell line. *Tylopilus* extracts or their isolated bioactive compounds could be considered for further study in pre-clinical experiments.

KEY WORDS: *Suillellus rubrosanguineus*; *Tylopilus felleus*; alkaloids; cytotoxicity; fungi

ABBREVIATIONS

DMEM: Dulbecco´s Modified Eagle´s Medium; **DMSO:** dimethyl sulfoxide; **FBS:** fetal bovine serum; **MTT:** 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; **PBS:** phosphate-buffered saline; **TylEtCap:** ethanol *Tylopilus* extract from the caps and stipes; **TylEtTub:** ethanol *Tylopilus* extract from the tubes; **TylChCap:** chloroform *Tylopilus* extract from the caps and stipes; **TylChTub:** chloroform *Tylopilus* extract from the tubes; **SuiEtCap:** ethanol *Suillellus* extract from the caps and stipes; **SuiEtTub:** ethanol *Suillellus* extract from the tubes; **SuiChTub:** chloroform *Suillellus* extract from the tubes

Introduction

There are only few studies focusing on the two members of the fungi family Boletace: *Suillellus rubrosanguineus* and *Tylopilus felleus* (Figure 1), commonly known as bitter bolete or bitter tylopilus. In the presented study we thus decided to investigate basic biological activities of these two fungus species. *Tylopilus felleus* grows in the whole Northern hemisphere and is common also in Slovakia, where it grows mainly in coniferous forests. *Tylopilus felleus* is typical for its bitter taste and pinkish hymenium. Due to the bitter taste, it is considered not edible (Šutara *et al.*, 2009). Only few studies about biological activities of *Tylopilus felleus* have been done (Grzybek *et al.*, 1994). This mushroom is also known in traditional Chinese medicine (Antonín *et al.*, 2013).

Suillellus rubrosanguineus (syn. Boletus rubrosanguineus, Rubroboletus rubrosanguineus) is a rare species, considered to be poisonous, which grows in middle and east Europe and on the Caucasus. In the Czech Republic it is included in the Red List of fungi in the category of critically endangered (Holec & Beran 2006). In Slovakia this mushroom grows rarely in higher altitudes in coniferous forests under *Picea abies* and *Abies alba*. Its occurrence differs from similar species such as *Boletus legalie, Boletus rhodoxanthus* and *Boletus satanas* (Šutara *et al.*, 2009). *Suillellus rubrosanguineus* is characteristic by its bright carmine colors on stipe and cap.

In this paper we focused on cytotoxicity profile of different extract preparations from *S. rubrosanguineus* and *T. felleus* in healthy and cancer cell lines and their analysis for the presence of alkaloids.

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Materials and methods

Materials

The Dulbecco´s Modified Eagle´s Medium (DMEM), penicillin-streptomycin mixture, phosphate-buffered saline (PBS), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Darmstadt, Germany). Dragendorff reagent, chloroform, ethanol, sulfuric acid, hydrochloric acid, ammonium, ether were obtained from Centralchem (Bratislava, Slovakia).

Specimens used in the study

Tylopilus felleus: Slovakia, Záhorská nížina, Holubičky, in coniferous forest, grass under *Pinus* trees; 19th of June, 2016; Leg. Drahomír Ďuriška et Det. Ondrej Ďuriška. *Suillellus rubrosanguineus:* Slovakia, Veľká Fatra, Valča; in mixed forest, under *Picea abies* trees; 3rd of July, 2016; Leg. et Det. Ondrej Ďuriška. Dr. Ondrej Ďuriška (co-author) made the photos of both collected fungi (Figure 1).

Extract preparations

Dried mushrooms samples were powdered in a blender before the extraction process. Mushroom samples were extracted by 96% ethanol or by concentrated chloroform for two weeks at occasional shaking. After evaporation of the solvents under reduced pressure, dry extracts were dissolved in DMSO and stored at –20 °C and used as mother liquor for all experiments. Separate extracts were prepared from stipes and caps (TylEtCap, TylChCap, SuiEtCap) and from hymenium (tubes) (TylEtTub, TylChTub, SuiEtTub, SuiChTub).

Cell cultures

NIH-3T3 (mouse embryonic fibroblast cells) were obtained as a gift from dr. Diana Vavrincová (Department of Pharmacology and Toxicology, Faculty of Pharmacy, Comenius University in Bratislava, Slovakia). MCF-7 (human breast adenocarcinoma cells) were donated by dr. Peter Gál (Department of Pharmacology, Faculty of Medicine, Pavol Jozef Šafárik University in Košice, Slovakia). Cells were grown at 37 °C in humidified atmosphere with 5% CO_2 in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were subcultured twice a week.

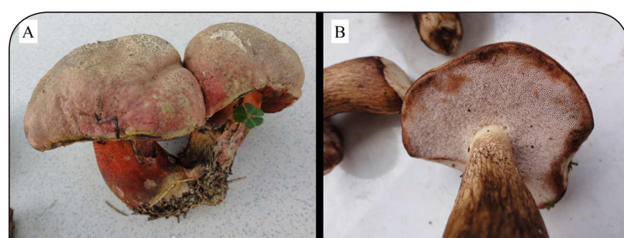

Figure 1. Photographs of collected fungi *Suillellus rubrosanguineus* (**A**) and *Tylopilus felleus* (**B**) made by dr. Ondrej Ďuriška (co-author).

In vitro analysis of cytotoxicity and cell proliferation

The effects of the compounds tested on the activity of mitochondrial dehydrogenases and proliferative functions of both cell lines were assessed using the reduction of tetrazolium salt MTT. The cells were seeded (10 000 cells/100 µL/well) in the 96-well-plate in complete medium. After 24 h, different concentrations of extracts, dissolved in complete medium, were added. The appropriate blanks were included into the experiment as well. The compound quercetin was used as a reference and it was dissolved in DMSO and then in complete medium. The final concentration of DMSO never exceeded 0.1%. Following 24-h incubation, a MTT solution was added to the wells (final concentration 0.4 mg/mL) except blank and after 4 h of incubation, the medium was removed and 100% DMSO was added to lyse the cells. The absorbance was measured (λ=570 nm) in Infinite M200 spectrofluorometer (Tecan, Switzerland). The amount of generated formazan (correlating to the number of viable and metabolically active cells) was calculated as a percentage of control cells and was set to 100%.

Qualitative determination of alkaloids

The presence of alkaloids in the extracts was identified with alkaloid-specific reagent Dragendorff, which creates red-orange precipitate with alkaloids. Dry chloroform extracts of fungi were dissolved in chlorform and were shaken out with 0.5 M sulfuric acid. The Dragendorff reagent was added into the water phase. Dry ethanol extracts of fungi were dissolved into the ether and a small amount of 15% ammonia was added. The solutions were shaken out with 0.1 M hydrochloric acid. The alkaloids were determined by adding a few drops of Dragendorff reagent. Extracts from leaves of *Atropa bella-donna* and from bark of *Cinchona succirubra* were employed as reference standards in tests of alkaloid detecting solutions.

Statistical analysis

Data were evaluated as means ± SEM from the three independent measurements. Comparisons among groups were made using the Student t-test with equal variance.

Results

Because of few data about cytotoxicity of two Boletaceae species, we provided MTT cytotoxicity tests on healthy mouse fibroblasts NIH-3T3 and on human cancer cell line MCF-7. We used the concentration range 6.25–100 µg/mL for *Suillellus rubrosanguineus* extracts and *Tylopilus felleus* extracts as well. The well-studied flavonoid quercetin was used as a reference with the concentrations from 6.25 to 50 µg/mL. The generation of formazan correlates directly with the number of viable cells with active mitochondrial reductases. The results are shown in Figure 2 (A, B, C).

Almost all higher concentrations of the four *Tylopilus* extracts caused significant decrease of the MTT reduction by cancer cells MCF-7 ($p<0.05$; $p<0.01$) (Figure 2 A). On

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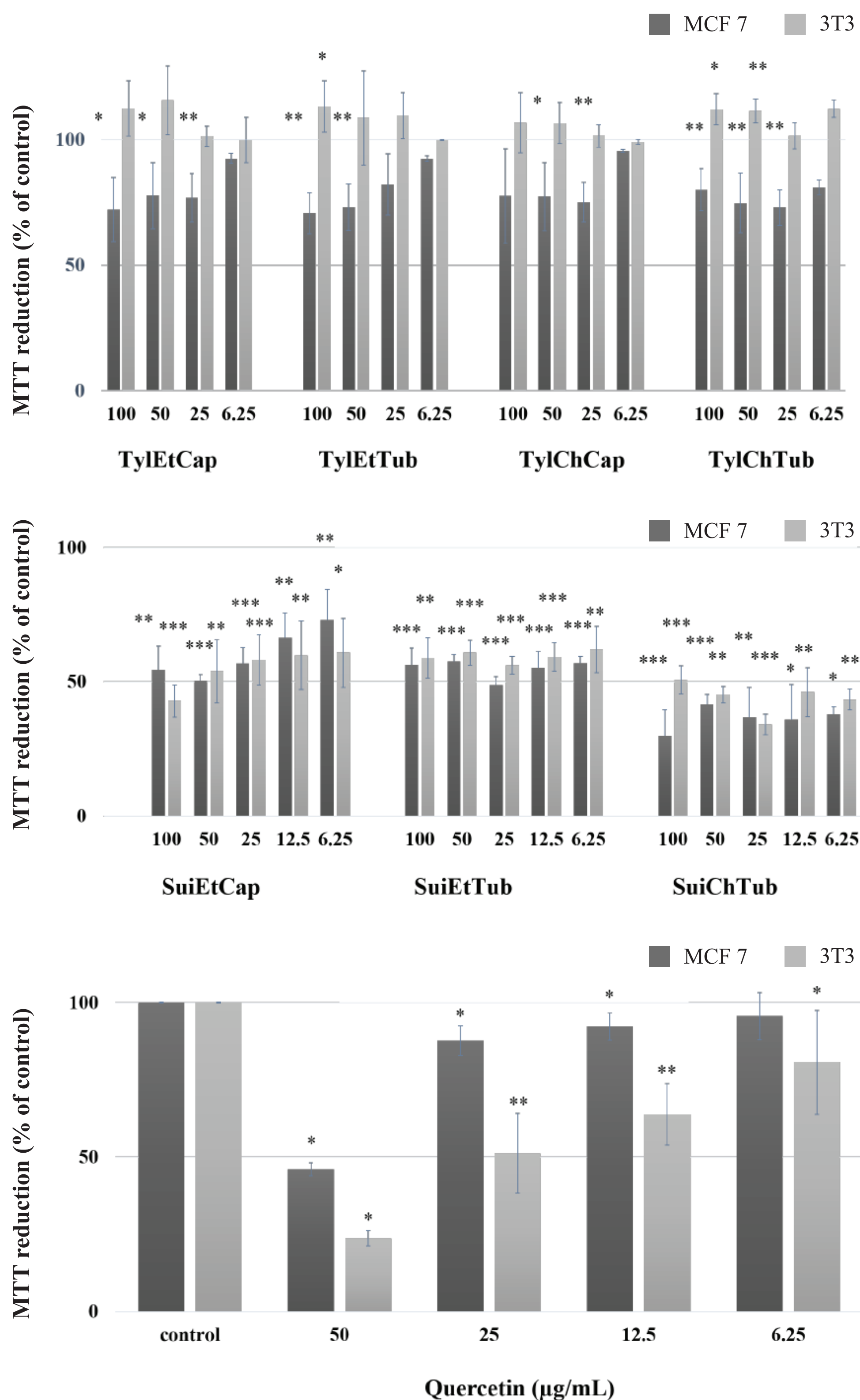

Figure 2. Comparison of viability/proliferation (assessed by mitochondrial reduction of MTT) of MCF-7 cells (dark gray) and 3T3 cells (light gray) after 24-h treatment with *Tylopilus* extracts (**A**), *Suillellus* extracts (**B**) and well-studied flavonoid quercetin used as a reference for set up the model (**C**) expressed as the percentage of control. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs*. control. TylEtCap – ethanol *Tylopilus* extract from the caps and stipes; TylEtTub – ethanol *Tylopilus* extract from the tubes; TylChCap – chloroform *Tylopilus* extract from the caps and stipes; TylChTub – chloroform *Tylopilus* extract from the tubes; SuiEtCap – ethanol *Suillellus* extract from the caps and stipes; SuiEtTub – ethanol *Suillellus* extract from the tubes; SuiChTub – chloroform *Suillellus* extract from the tubes.

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the contrary, higher concentrations of the chloroform and ethanol extracts of the tubes caused significant increase of the viability of NIH-3T3 ($p<0.05$; $p<0.01$) and extracts from the caps and stipes caused mild enhancement of the viability and/ or proliferation of the NIH-3T3 cells.

In general, the extracts from *Suillellus rubrosanguineus* are significantly more cytotoxic than the extracts from *Tylopilus felleus.* All three *Suillellus* extracts in the concentrations used significantly decreased the metabolic activity or/and proliferation of both used cell lines ($p<0.05$; $p<0.01$; $p<0.001$) (Figure 2 B). The chloroform extract from the tubes of *Suillellus rubrosanguineus* is more toxic than the two ethanol extracts tested. There is no significant difference in the effects of the three extracts of *Suillellus rubrosanguineus* on NIH-3T3 compared to MCF-7.

The flavonoid quercetin used as a reference (Figure 2 C) showed higher cytotoxicity to healthy NIH-3T3 than to cancer cell line MCF-7. Our results for quercetin cytotoxicity are in accordance with previous *in vitro* studies (Chou *et al.,* 2010; Danihelova *et al.,* 2013). These results serve to ensure that this method is set correctly.

In our study we detected the presence of alkaloids by alkaloid-specific Dragendorff reagent, which creates red-orange precipitates with them (Table 1). All extracts from both fungi reacted positively with the Dragendorff reagent, the ethanol extracts showed stronger reactions than the chloroform extracts. The alkaloids are localized in the tissues of caps as well as tubes of fungi.

Table 1. Response of the tested extracts to specific alkaloid indicator.

Extracts	Reaction with Dragendorff reagent
TylEtCap	***
TylEtTub	***
TylChCap	**
TylChTub	**
SuiEtCap	***
SuiEtTub	***
SuiChTub	**

From the extracts the alkaloid cations were released and they could make positive reaction with Dragendorff reagent as a red precipitate.
*** intense reaction; ** moderate reaction; * weak reaction; – no reaction (range inspired by Furr & Mahlberg, 1981).

Discussion

So far, only a few studies concerning biological activities of the two fungi of Boletaceae family *Suillellus rubrosanguineus* and *Tylopilus felleus,* which grow in the forests of Middle Europe, were done.

For this reason we focused on this topic. Our research includes basal cytotoxicity test on healthy and cancer cell lines and qualitative determination of the presence of alkaloids.

In general, the high selectivity of the plant extracts or synthetic compounds for cancer cell lines is preferred. Well-known cytostatics paclitaxel, doxorubicin, tamoxifen have inhibition concentrations IC_{50} more times lower for cancer cell lines than for normal cells (Hasanpourghadi *et al.,* 2017). Prsumably, no studies about the cytotoxicity on the healthy or cancer cell lines of the extracts from the mentioned two fungi have been done. Only some cytotoxicity studies of the β-glucan tylopilan isolated from *Tylopilus felleus* were provided. Tylopilan showed antitumor activity on 180-TG Crocker cells in the concentration range 300–37.5 μg/mL (Grzybek *et al.,* 1994). In general, β-glucans are natural polysaccharides present in plants, fungi, yeast, bacteria and algae. Several studies indicate that β-glucans could activate cells of the immune system against pathogens or against cancer cells, as well as exert direct cytostatic, antibacterial and antiviral activities and regenerative effects (Browder *et al.,* 1990; Markova *et al.,* 2003; Baldwin *et al.,* 2015).

Our results demonstrate that only *Tylopilus* extracts exhibit selective cytotoxicity to cancer cells over healthy non-tumorigenic cells in pharmacological concentrations. Similar results have been seen by Jafaar *et al.* (2014) with β-glucan, which at higher concentrations acted toxic to breast cancer cells but promoting proliferation to healthy cells. Lentinan, β-glucan isolated form the mushroom *Lentinus edodes*, selectively inhibited proliferation of breast cancer cells and showed good safety profile in normal cells (Xu *et al.,* 2017). This shows the worth of continuing in the following cytotoxicity tests. Precisely because our experiments proved only mild toxicity of extracts to cancer cells, further tests could be done with higher concentratons of extracts, or with extract isolates to achieve higher toxicity to cancer cells while preserving the preferred selectivity.

The extracts from *Suillellus rubrosanguineus* have proven to be significantly more cytotoxic than extracts from *Tylopilus felleus* to both cell lines in the concentration range used. These results could contribute to the knowledge about known poisonousness of the mushroom *S. rubrosanguineus*.The mushroom *Tylopilus felleus* is generally considered to be non-edible due to its bitter taste.

The presence of alkaloids in the fungi could contribute to the whole image of toxicity. Alkaloids are naturally occurring organic nitrogen-containing bases of plants as well as fungi. They have diverse biological effects on humans and animals in very low concentrations and toxic effects with the higher concentrations (Nugroho *et al.,* 2015; Bun *et al.,* 2008).

Probably no data are known about quantitative or qualitative characterization of alkaloids in fungi *Suillellus rubrosanguineus* and *Tylopilus felleus.* Some alkaloids have been identified in some species of the Boletaceae family (Mahmood *et al.,* 2010).

In the present study, we provided some novel additional data concerning the biological profile of less biomedically studied but commonly known two fungi species of the Boletaceae family *Suillellus rubrosanguineus* and *Tylopilus felleus.* The results show beneficial specificity

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of cytotoxicity of the four different *Tylopilus* extract preparations. They are cytotoxic for human breast cancer cells MCF-7 and they caused slightly enhanced proliferation/ metabolic activity in mouse fibroblasts NIH- 3T3 in pharmacological concentrations. The *Suillellus* extracts showed greater cytotoxicity than *Tylopilus* extracts, however, with a comparable extent in both cell lines. The presence of alkaloids was found in all extracts, which could contribute to their cytotoxicity. The extracts of *Tylopilus felleus* showed biological activities, which could open a perspective for future detailed study focusing, for instance, on their potential use in adjuvant therapy of cancer.

Acknowledgments

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REFERENCES

Antonín V, Jablonský I, Šašek V, Vančuříková Z. (2013). *Huby ako liek*. Ottovo nakladateľstvo, Martin.

Baldwin K T, Carbajal K S, Segal B M, Giger R J. (2015). Neuroinflammation triggered by β-glucan/dectin-1 signaling enables CNS axon regeneration. *Proc Natl Acad Sci U S A* **112**(8): 2581–6.

Browder W, Williams D, Pretus H, Olivero G, Enrichens F, Mao P, Franchello A. (1990). Beneficial effect of enhanced macrophage function in the trauma patient. *Ann Surg* **1**: 605–613.

Bun S, Laget M, Chea A, Bun H, Ollivier E, Elias R. (2008). Cytotoxic activity of alkaloids isolated from *Stephania rotunda in vitro,* cytotoxic activity of cepharanthine. *Phytother Res* **23**(4): 587–590.

Danihelova M, Veverka M, Sturdik E, Jantova S. (2013). Antioxidant action and cytotoxicity on HeLa and NIH-3T3 cells of new quercetin derivatives. *Interdiscip Toxicol* **6**(4): 209–216.

Furr M, Mahlberg P G. (1981). Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa*. *J Nat Prod* **44**(2): 153–159.

Grzybek J, Zgorniak-Nowosielska I, Kasprowicz A, Zawilinska B, Kohlmunzer S. (1994). Antitumor activity of a fungal glucan tylopilan and *Propionibacterium acnes* preparation. *Acta Soc Bot Pol* **63**: 3–4.

Hasanpourghadi M, Pandurangan AK, Karthikeyan C, Trivedi P, Mustafa MR. (2017). Mechanisms of the anti-tumor activity of Methyl 2-(-5-fluoro-2-hydroxyphenyl)-1H-benzo[d]imidazole-5-carboxylate against breast cancer *in vitro* and *in vivo*. Oncotarget **8**(17): 28840–28853.

Holec J, Beran M, *et al.* (2006). *Červený seznam hub (makromycetů) České republiky*. Příroda, Praha.

Chou Ch, Yang J, Lu H, *et al.* (2010). Quercetin-mediated Cell Cycle Arrest and Apoptosis Involving Activation of a Caspase Cascade through the Mitochondrial Pathway in Human Breast Cancer MCF-7 Cells. *Arch Pharm Res* **33**: 1181–1191.

Jafaar Z M T, Litchfield L M, Ivanova M M, Radde B N, Al-Rayyan N, Klinge C M. (2014). β-D-glucan inhibits endocrine-resistant breast cancer cell proliferation and alters gene expression. *Int J Oncol* **44**(4): 1365–1375.

Mahmood Z A, Ahmed S W, Azhar I, Sualeh M, Baig M T, Zoha S. (2010). Bioactive alkaloids produces by fungi I. Updates on alkaloids from the species of the genera *Boletus*, *Fusarium* and *Psilocybe. Pak J Pharm* **23**(3): 349–357.

Markova N, Kussovski V, Drandarska S N, Georgieva N, Radoucheva T. (2003). Protective activity of lentinan in experimental tuberculosis. *Int Immunopharmacology* **3**: 1557–1562.

Nugroho A E, Akbar F F, Wiyani A, Sudarsono. (2015). Cytotoxic Effect and Constituent Profile of Alkaloid Fractions from Ethanolic Extract of *Ficus septica* Burm. f. leaves on T47D Breast Cancer Cells. *Asian Pac J Cancer Prev* **16**(16): 7337–7342.

Šutara j, Mikšík M, Janda V. (2009). *Hřibovité houby*. Academia, Praha.

Xu H, Zou S, Xu X. (2017). The β-glucan from Lentinus edodes suppresses cell proliferation and promotes apoptosis in estrogen receptor positive breast cancers. *Oncotarget* **8**(49): 86693–86709.

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ORIGINAL ARTICLE

In vivo analysis of Bisphenol A induced dose-dependent adverse effects in cauda epididymis of mice

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ABSTRACT

Bisphenol A is widely used as a material for the production of epoxy resins and polycarbonate plastics. It contaminates various food stuffs by getting leached out from their container lining. Limited information is available on its effects on the male reproductive system. The aim of the present study was to evaluate the extent to which bisphenol A can affect the reproductive system by measuring biochemical and histological changes in the epididymis. Inbred Swiss strain male albino mice were orally administered 80, 120 and 240 mg/kg body weight/day of BPA for 45 days. After completion of treatment, the animals were sacrificed; cauda epididymis was isolated, weighed, used for biochemical and histopathological studies. The results revealed that BPA administered for 45 days caused significant ($p<0.05$) and dose-dependent reduction in epididymis weight. There was significant ($p<0.05$) increase in lipid peroxidation and the acid phosphatase activity. Dose dependent reduction in protein, sialic acid contents, as well as the activity of enzymatic antioxidants and mitochondrial enzymes was recorded compared to vehicle treated group. The effect was dose-dependent. Histopathological alteration was observed. This study concludes that BPA causes toxicity in epididymis of mice by generating free radicals, which may be a possible reason for reduction in sperm parameters.

KEY WORDS: bisphenol A; cauda epididymis; biochemical study; *in vivo* toxicity

ABBREVIATIONS

BPA: bisphenol A; **LPO:** lipid peroxidation; **MD:** medium dose; **LD:** low dose; **HD:** high dose; **ROS:** reactive oxygen species; **ACP:** acid phosphatase, **CAT:** catalase; **SDH:** succinate dehydrogenase; **T.S:** transverse section

Introduction

The use of plastics has become one of the defining characteristics of modern life. But many of the plastic products people use on a daily basis contain components that can prove harmful to human health and the environment. One such component is a chemical called bisphenol A [2,2-bis(4-hydroxyphenyl)propane], It is one of the highest volume chemicals produced worldwide (Vandenberg *et al.,* 2010). It is a key monomer in the production of epoxy resins and polycarbonate plastic used in manufacture of many household products (Staples *et al.,* 1998; Ranjit *et al.,* 2010). As epoxy resins are used as coatings inside of almost all food and beverage cans, it leaches into food and thus food is considered the main source of its exposure (Vandenberg *et al.,*2007; Carwile *et al.,* 2009).

Bisphenol A mimics estrogen activity that interferes with the hormonal system in animals and human beings and contributes to adverse health effects (Rochester *et al.,* 2013). It induces hepatic damage and mitochondrial dysfunction by increasing oxidative stress in the liver and other vital organs (Sangai & Verma, 2012; Hassan *et al.,* 2012; Chen *et al.,* 2012; Xia *et al.,* 2014, Elswefy *et al.,* 2016). A study carried out by Verma and Sangai (2009) showed that treatment with bisphenol A causes cytotoxicity in human erythrocytes, which may be due to oxidative stress. However, its effects on the epididymis, highly specialized tissue responsible for maturation and storage of spermatozoa, is not understood.

According to our previous study (Samova *et al.,* 2016), BPA treatment for 45 days causes significant ($p<0.05$), dose-dependent decrease in sperm count (r=0.992:

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BPA-LD 80.86%; BPA-MD 47.89%; BPA-HD 25.60%), sperm motility (r=0.995: BPA-LD 73.48%; BPA-MD 51.78%; BPA-HD 24.78%) and sperm viability (r= 0.996: BPA-LD 76.07%; BPA MD 55.85%; BPA HD 25.05%) as compared to vehicle treated control.

As there was reduction in sperm functional parameters, our aim was to investigate what changes affect sperm function. We investigated the sub-chronic toxic effect of bisphenol A in the cauda epididymis of mice by analyzing the oxidative stress, enzymatic antioxidants, energy metabolism, and total contents.

Materials and Methods

Chemicals

Bisphenol A was procured from Hi Media Laboratories Pvt. Ltd., Mumbai, India. Olive oil was obtained from Figaro, Madrid, Spain. All the other chemicals used in the present study were of analyzer grade reagent.

Experimental animals

In this study, inbred healthy adult Swiss strain male albino mice weighing 30–35 g were obtained from Cadila pharmaceutical Center, Ahmedabad, India. Animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India. They were housed in an air-conditioned room at a temperature of 22±3 °C and 45–55% relative humidity with a 12 h light/dark cycle throughout the experiment. The animals were fed certified pelleted rodent food supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and potable water ad libitum. All the experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experiment on Animals (Reg.-167/1999/CPCSEA), New Delhi, India. Animals were handled according to the guidelines published by the Indian National Science Academy, New Delhi, India (1991).

Experimental design

Dose selection

Different doses of BPA were selected on the basis of LD50 value (Kimura *et al.,* 2007). Animals of BPA-treated groups received three different doses of bisphenol A, *i.e.* 1/10th, 1/20th and 1/30th of LD50 (240,120 and 80 mg/kg bw/day respectively) for 45 days.

Experimental protocol

The experimental protocol is shown in Table 1.The 50 mice were randomly divided into five groups each containing 10 animals. Animals of Group I (untreated control) were kept without any treatment and given free access to food and water. Group II (vehicle control) animals were treated with olive oil (0.2 ml/animal/day), as olive oil was used to dissolve bisphenol A. Animals of group III, IV and V received three respectively different doses of BPA (80, 120 and 240 mg/kg bw/day) for 45 days.

All treatments were given orally using a feeding tube attached to a hypodermic syringe.

Necropsy

After treatment, the animals were sacrificed using anesthetic ether. The epididymis was dissected out quickly, blotted free from blood and used for the histopathological study and biochemical parameters such as lipid peroxidation, protein and sialic acid contents, as well as the activities of enzymatic antioxidants, acid phosphatase and mitochondrial enzymes (ATPase and succinate dehydrogenase).

Biochemical parameters

Lipid Peroxidation

The levels of lipid peroxidation were measured as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa *et al.* (1979) with modifications. The results were expressed as nano moles of MDA per gram of protein.

Enzymatic antioxidants

Catalase (E.C.1.11.1.6) activity was analyzed using the method described by Luck (1963), utilizing hydrogen peroxide as substrate. Decrease in absorption was noted at 240 nm. The enzyme activity was expressed as µmoles H2O2 consumed/mg protein/min. Superoxide dismutase (E.C.1.15.1.1) activity (SOD) was measured by the method of Kakkar *et al.* (1984). The enzyme activity was expressed as units/mg protein. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of $O_2^{\bullet}$. The glutathione peroxidase (E.C.1.11.1.9) activity (GSH-Px) in the testis was assayed by the modified method of Paglia and Valentine (1967). The decrease in absorbance was recorded for 3 min at 340 nm. The enzyme activity was expressed as units/mg protein/min, where 1 unit of GSH-Px equals to nmoles of NADPH consumed/mg protein/min.

Energy metabolism and phosphatase activity

The adenosine triphosphatase (ATPase) activity in the cauda epididymis was assayed by the method of Quinn and White (1968). The enzyme activity was expressed as µmoles inorganic phosphate released/mg protein/30 min. Succinic dehydrogenase (SDH) activity was assayed by the method of Beatty *et al.* (1966). The enzyme activity was expressed as µg formazon formed/mg protein/15 min. The acid phosphatase (ACP) activity was assayed by the method as described in Sigma Technical Bulletin (Sigma Technical Bulletin, MO, USA. 2001). The enzyme activity was expressed as µmoles *p*-nitrophenol released/mg protein/30 min.

Total content estimation

Protein content in the cauda epididymis was estimated by the method of Lowry *et al.* (1951). The protein content was expressed as mg/100 mg tissue weight. The concentration of sialic acid was assessed by the method of Jourdian *et*

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al. (1971). The sialic acid content was expressed as µg/mg tissue weight.

Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) followed by Tukey's test using Graph Pad Instant software version 5.03. Data are expressed as the means ± S.E.M. The level of significance was accepted at $p<0.05$. Pearson's correlation analysis was used to determine the correlation between controls and treated animals.

Histopathological studies

Histopathological studies were carried out using the standard technique of hematoxylin and eosin staining. The cauda epididymis of all control and treated groups of animals were dissected out, blotted free of blood and fixed in Bouin's solution immediately after autopsy. The preserved tissues were dehydrated by passing through ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58 to 60 °C). Sections of 5 µm were cut on a rotary microtome and stained with H & E, dehydrated in alcohol, cleared in xylene, mounted in DPX and examined under a light microscope.

Results

Absolute and relative weights

Table 2 shows results of BPA treatment on absolute and relative weights of cauda epididymis. No significant changes were observed in absolute and relative weights of cauda epididymis between different control groups of animals (Groups I–II). The treatment of BPA (Groups III–V) for 45 days caused significant ($p<0.05$) reduction in absolute and relative weights of cauda epididymis as compared to vehicle control group of animals (Group II). These effects were dose-dependent (r=0.940, 0.891 respectively). The maximum reductions in absolute and relative weights were up to 33.84% and 30.16% respectively.

Lipid peroxidation and activity of enzymatic antioxidants

The effect of BPA treatment on lipid peroxidation as well as enzymatic antioxidants in cauda epididymis is shown in Table 3. No significant difference was noted in LPO and enzymatic antioxidants between different control groups of animals (Groups I–II). Oral administration of BPA (Groups III–V) for 45 days caused significant ($p<0.05$), dose-dependent (r=0.876) increase in LPO (LD: 42.34%, MD: 68.43% and HD: 102.14%) as compared to vehicle control (Group II). The activities of enzymatic antioxidants such as CAT, SOD and GSH-Px were significantly lowered in BPA-treated mice as compared to vehicle control. These effects were in dose-dependent manner (r=0.870, 0.864, 0.770, respectively). The maximum reduction was observed with BPA-HD (Figure 1).

Mitochondrial enzymes and phosphatase activities

BPA-induced changes in biochemical parameters in cauda epididymis are shown in Table 4. No significant difference was noted in activities of ACP, ATPase and SDH between different control groups of animals (Groups I–II). Similarly, as compared to vehicle control, BPA treatment caused significant ($p<0.05$), dose-dependent (r=0.929) increase in ACP activity (LD: 58.98%, MD: 151.862% and LD: 201.71%), as compared to vehicle control (Figure 2).

BPA treatment also caused significantly ($p<0.05$) decreased activities of ATPase and SDH. The effect was dose-dependent (r=0.944, 0.919, respectively).

Total protein and ascorbic acid

Reduction in total content is shown in Table 5. Oral administration of BPA (Groups III–V) caused significant ($p<0.05$) decrease in protein (BPA-LD: 31.84%, BPA-MD: 50.14%, BPA-HD: 64.91%,) and sialic acid (BPA-LD: 20.79%, BPA-MD: 31.33%, BPA-HD: 44.43%) contents as compared to vehicle treated control group (Group II). These effects were dose-dependent (r=0.942, 0.948) (Figure 3).

Histopathological analysis

The cauda epididymis of all control groups (Groups I–II) of animals showed normal tubules with pseudostratified epithelium lined by stereocilia and containing dense sperm bundles in the lumen of the tubules (Figure 4) However, BPA treatment (Groups III–V) for 45 days resulted in alterations in the cauda epididymis. Bisphenol A treatment caused degeneration in epithelium, decrease in stereo cilia, and reduction in sperm density and wider space between tubules (Figure 5). The effect was more pronounced in BPA-HD-treated group (Group V).

Table 1. Experimental protocol.

Sr. No.	Experimental Groups	Number of animals	Duration of treatment (Days)	Day of necropsy
Control groups				
I	Untreated control	10	45	46th
II	Vehicle control (0.2 ml olive oil/animal/day)	10	45	46th
BPA-treated groups				
III	BPA-Low dose (80 mg/kg body weight/day)	10	45	46th
IV	BPA-Medium dose (120 mg/kg body weight/day)	10	45	46th
V	BPA-High dose (240 mg/kg body weight/day)	10	45	46th

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Table 2. Bisphenol A induced changes in absolute and relative weight of cauda epididymis of mice.

Sr. No.	Experimental groups	Absolute weight	Relative weight
Control groups			
I	Untreated control	24.12 0.26	61.53±0.39
II	Vehicle control (0.2 ml olive oil/animal/day)	24.08±0.16	61.48±0.71
BPA-treated groups			
III	BPA-Low dose (80 mg/kg bodyweight/day)	21.32±0.34* (11.44)	54.99±0.87* (10.54)
IV	BPA-Medium dose (120 mg/kg bodyweight/day)	18.54±0.27* (22.98)	49.54±1.06* (19.56)
V	BPA-High dose(240 mg/kg bodyweight/day)	15.98±0.28* (33.84)	42.94±1.00* (30.16)

Values are mean±S.E.M., n=10. Values shown in parenthesis indicate: Brackets – Percent change in BPA-treated from vehicle treated control group. Significance at the level of *p<0.05, as compared with vehicle control group. No significant difference was noted between untreated and vehicle control group. Units: Absolute weight – mg; Relative weight – mg/100 gm body weight.

Table 3. Bisphenol A-induced changes on lipid peroxidation and enzymatic antioxidants in cauda epididymis of mice

Sr. No.	Experimental group	LPO	Enzymatic Antioxidants		
			Catalase	SOD	GSH-Px
Control groups					
I	Untreated control	1.462±0.11	2.066±0.05	6.725±0.31	0.407±0.03
II	Vehicle control (0.2 ml olive oil /animal/day)	1.470±0.09	2.022±0.05	6.707±0.28	0.402±0.02
BPA-treated groups					
III	BPA-Low dose (80mg/kg bodyweight/day)	2.092±0.12* (42.33)	1.643±0.07* (18.75)	5.648±0.18* (15.79)	0.310±0.02* (22.90)
IV	BPA-Medium dose (120 mg/kg bodyweight/day)	2.476±0.10* (68.43)	1.309±0.05* (35.24)	4.911±0.12* (26.78)	0.259±0.01* (35.53)
V	BPA-High dose (240 mg/kg bodyweight/day)	2.971±0.06* (102.14)	1.111±0.12* (45.04)	4.021±0.11* (40.05)	0.209±0.02* (47.99)

Values are mean±S.E.M., n=10, Values shown in parenthesis indicate: Brackets – Percent change in BPA-treated from vehicle control. Level of significance; *ap* <0.05 as compared to vehicle control, *bp*<0.05 as compared to BPA-HD -treated. No significant difference was noted between different control groups (Groups I-III). Units: LPO – nmoles MDA formed/mg protein/60 min; Catalase – µmoles H2O2 consumed/mg protein/min; SOD – units/mg protein; GSH-Px- nmoles of NADPH consumed/mg protein/min.

Table 4. Bisphenol A induced changes in phosphatase activity and energy metabolism in cauda epididymis of mice.

Sr. No.	Experimental groups	Phosphatase activity (ACP)	Energy Metabolism	
			ATPase	SDH
Control groups				
I	Untreated control	0.46±0.06	1.62±0.02	19.21±0.26
II	Vehicle control (0.2 ml olive oil/animal/day)	0.45±0.01	1.61±0.01	19.17±0.36
BPA-treated groups				
III	BPA-Low dose (80 mg/kg bodyweight/day)	0.61±0.01* (58.98)	1.40±0.02* (13.16)	15.33±0.26* (19.96)
IV	BPA-Medium dose (120 mg/kg bodyweight/day)	1.00±0.02* (151.862)	1.22±0.02* (24.31)	13.34±0.48* (30.38)
V	BPA-High dose (240 mg/kg bodyweight/day)	1.16±0.05* (201.71)	1.07±0.02* (33.80)	1073±0.43* (43.99)

Values are mean±S.E.M., n=10. Values shown in parentheses indicate: Brackets – Percent change in BPA treated from vehicle treated control group. Significance at the level of *p<0.05, as compared with vehicle control,No significant difference was noted between untreated and vehicle control groups. Units: ACP – µmoles p-nitrophenol released/mg protein/30 min; ATPase – µmoles inorganic phosphate released/mg protein/30 min; SDH – µg formazon formed/mg protein/15 min

Discussion

The epididymis is an important organ in the male reproductive system in which the testicular spermatozoa undergo maturation (Cornwall 2009). Sperm maturation depends on the secretary product of the epididymis. The epididymis provides a luminal microenvironment for sperm maturation and storage under androgen control (Robaire *et al.,* 1988; Serre & Robaire 1998). Previous studies showed BPA to cause generation of free radicals (Chitra *et al.,* 2003; Kabuto *et al.,* 2003).

In the present study, BPA was administered in order to evaluate its effect through a complete spermatogenic cycle, which takes approximately 45 days in mice (Clermont, 1972; Hess *et al.,* 2009) and the length of spermatogenic cycle is considered as biological constant

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Table 5. Bisphenol A induced changes in total contents in cauda epididymis of mice.

Sr. No.	Experimental groups	Protein	Sialic acid
Control groups			
I	Untreated control	12.28±0.58	10.03±0.16
II	Vehicle control (0.2 ml olive oil/animal/day)	12.17±0.39	10.04±0.13
BPA-treated groups			
III	BPA-Low dose (80 mg/kg bodyweight/day)	8.295±0.16* (31.84)	7.95±0.15* (20.79)
IV	BPA-Medium dose (120 mg/kg bodyweight/day)	6.07±0.81* (50.14)	6.89±0.16* (31.33)
V	BPA-High dose (240 mg/kg bodyweight/day)	4.27±0.17* (64.91)	5.58±0.14* (44.43)

Values are mean±S.E.M., n=10. Values shown in parentheses indicate:Bracket – Percent change in BPA-treated from vehicle treated control group. Significance at the level of *p<0.05, as compared with vehicle control, No significant difference was noted between untreated and vehicle control groups. Units: Protein – mg/100 mg tissue weight; Sialic acid – μg/mg tissue weight.

controlled by germ cell, thus we exposed mice to BPA for 45 days. Gravimetric analysis revealed that BPA treatment for 45 days caused dose-dependent significant decrease in absolute and relative weights of cauda epididymis (Chitra *et al.*, 2003). Vom Saal *et al.* (1998) and Delclos *et al.* (2014) also reported that decreases in epididymis weights were associated with degenerative changes with hypospermia. Histopathology showed reduction in sperm bundles (Chitra *et al.*, 2003; 2001). Reduction in weights of cauda epididymis could be due to degenerative changes in epithelium and lower sperm concentration in lumen. Moreover, it may be due to the inhibition of spermatogenesis, decreased elongated spermatids and steroidogenic enzyme activity (Takahashi & Oishi, 2001; Lanning *et al.*, 2002).

Overproduction of reactive oxygen species (ROS) and free radicals constitutes oxidative stress that can be detrimental to sperm as associated with male fertility (Agarwal *et al.*, 2014). If spermatozoa are exposed to excessive levels of ROS, their fertilizing capacity and genetic integrity could be compromised (Aitken *et al.*, 2014; 2016). Oral administration of BPA generates oxidative stress (ROS) which damages the lipid membrane and moreover reduces the activities of enzymatic antioxidants in cauda epididymis of mice. BPA exerts some of its effects by binding to the nuclear steroid receptors for estrogen to subsequently impact expression of estrogen-responsive gene products (Wetherill *et al.*, 2007). Moreover, the study by Qiu *et al.* indicates that a low BPA concentration can induce spermatogenesis disorders mainly through decreasing androgen receptor expression (Qiu *et al.*, 2013). Unfavorable condition for sperm leads to deterioration of the fertility rate (Gabrielsen *et al.*,2016). El-Missiry *et al.* (2014) explained that bisphenol A elicits depletion of antioxidant defense system and induces oxidative stress. Another study done by Hassan *et al.* (2012) reported that bisphenol A induces hepatotoxicity through oxidative stress and ultimately decreases the antioxidant enzymes. Catalase and glutathione peroxidase are important enzymes of antioxidant defense systems, which protect tissue against oxidative stress induced by reactive oxygen species (Lei *et al.*, 2016). Both these enzymes catalyze the

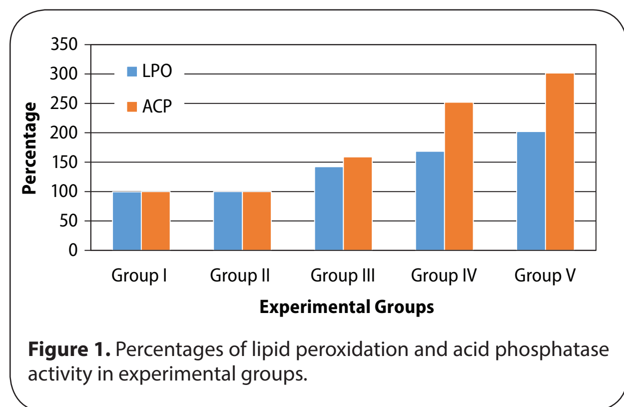

Figure 1. Percentages of lipid peroxidation and acid phosphatase activity in experimental groups.

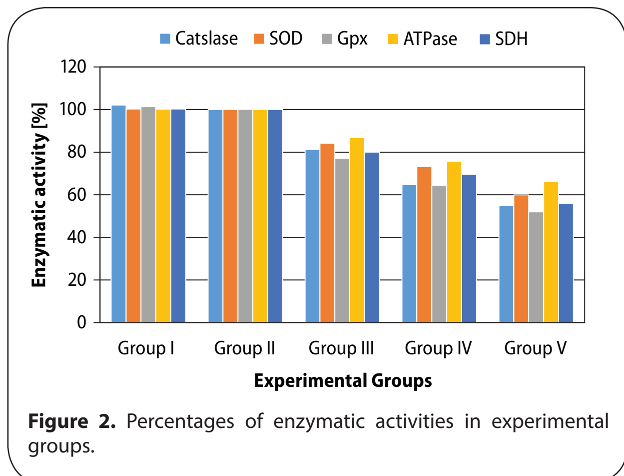

Figure 2. Percentages of enzymatic activities in experimental groups.

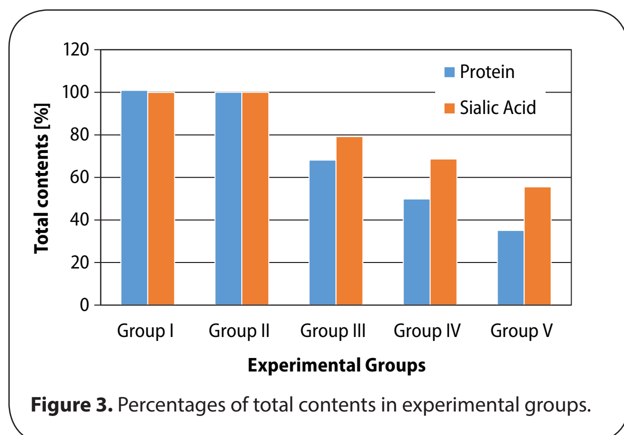

Figure 3. Percentages of total contents in experimental groups.

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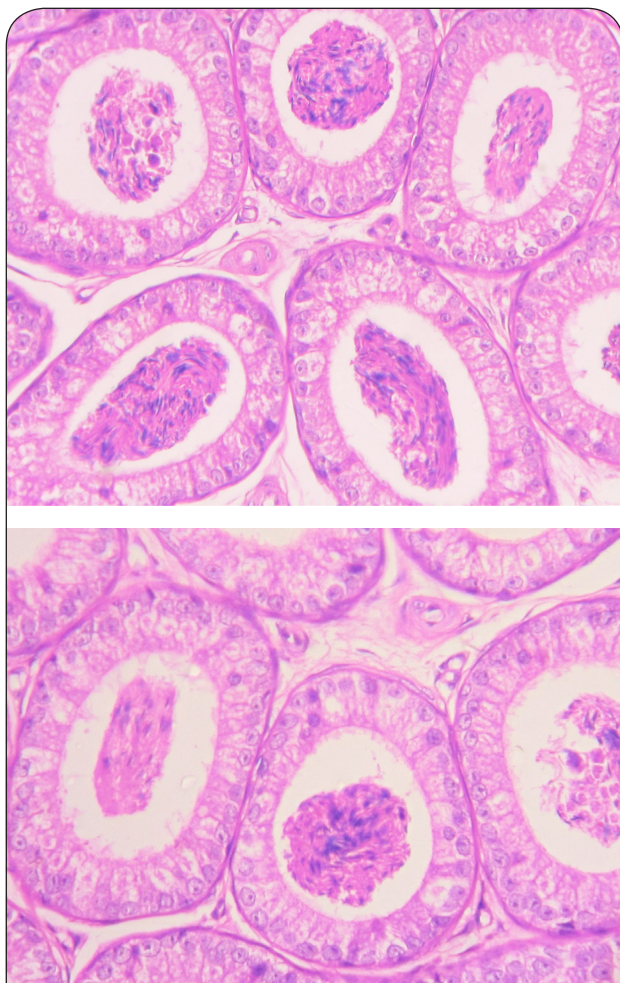

Figure 4. T.S. of cauda epididymis of untreated control mice (group I-II) showing normal tubules with pseudostratified epithelium lined by stereocilia and containing dense sperm bundles in the lumen of the tubules (H & E staining, 400×).

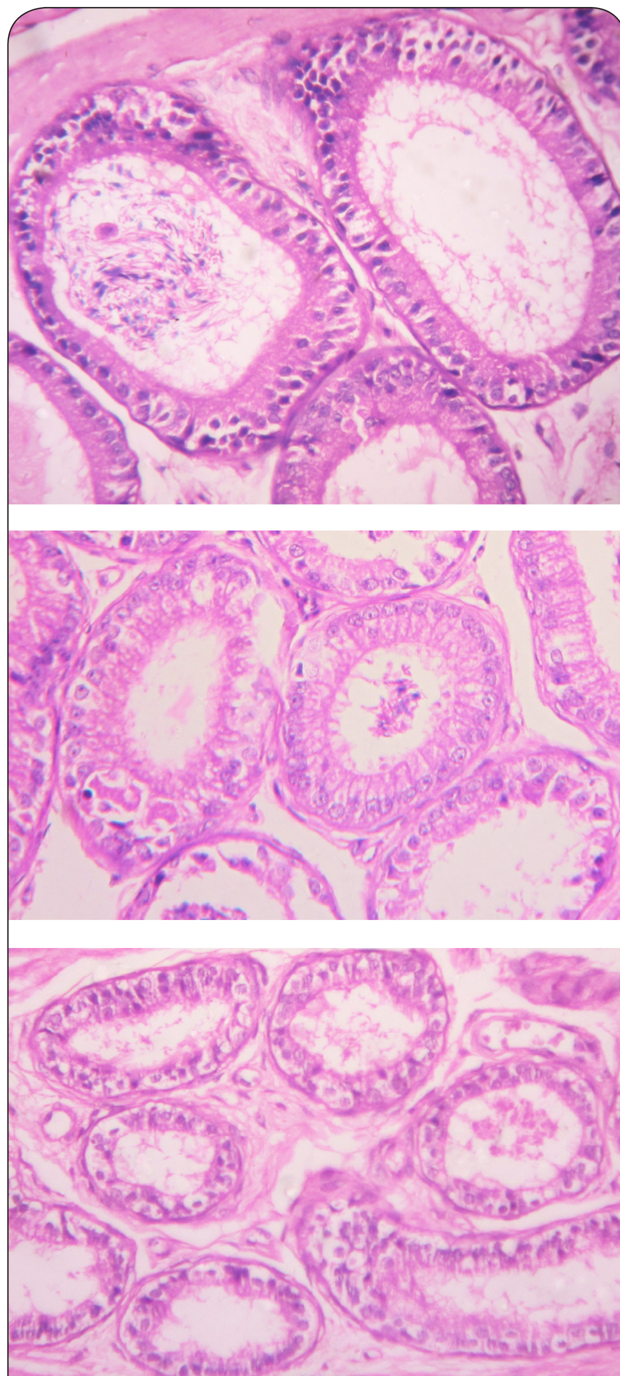

Figure 5. T.S. of cauda epididymis of BPA-HD-treated mice (group III-V) showing degenerated epithelium with decrease in stereocilia, reduction in sperm density and wider space between tubules as per increases the exposure of BPA (H & E staining, 400×).

hydrolysis of H2O2 into water and oxygen molecules to prevent tissue injury (Prescott *et al.,* 2016).

The cell has numerous defense mechanisms to fight against oxidative stress. Our result shows the decrease in activity of CAT. This indicates that H2O2 was most probably present in high levels, moreover CAT is involved predominantly in the detoxification of high H2O2 levels. Reduction in the activity of catalase may reflect incapability of mitochondria and eliminate hydrogen peroxide produced after exposure to BPA (Bindhumol *et al.,* 2003).

The present study shows that exposure to BPA causes significant, dose-dependent decrease in energy metabolism. ATPase is required for enzymatic hydrolysis of ATP, which is important for intracellular transfer of energy (Zeisel, 2012). Activity of SDH, also significantly decreased in cauda epididymis by BPA administration. SDH is a key enzyme of mitochondrial Krebs cycle and it is mainly concerned with aerobic oxidation of acetylCoA and generation of ATP (Iacobazzi *et al.,* 2014). Of the Krebs cycle dehydrogenases, SDH is more active than any other enzyme (Putilina *et al.,* 1969). Thus reduction in ATPase and SDH activity could be due to alteration in mitochondria and this could lead to reduction in sperm viability and motility (Ramalho-Santos *et al.,* 2009; Piomboni *et al.,* 2012). Oral administration of bisphenol A for 45 days caused significant dose dependent reduction in activities of SDH and ATPase in epididymis of mice. The effect was comparatively more pronounced in high-dose bisphenol A-treated group than with a low dose. These results indicate that the onset of cytotoxicity caused by

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BPA may depend on the intracellular energy status and that mitochondria are important targets of the compound (Amaral *et al.,* 2016). The toxicity caused by inhibition of ATP synthesis may be related to the concentration of unmetabolized free BPA remaining in the cell suspensions. Oral administration of BPA for 45 days caused dose-dependent significant increase in ACP activity in cauda epididymis of mice (Graph.1). Acid phosphatase is a marker enzyme for the lysosomal integrity and important for tissue reorganization and tissue repair (Collins and Lewis *et al.,* 1971, Gómez-Sintes *et al.,* 2016). Increased levels of ACP show the tissue damage or nercosis, which is seen in histopathology (Fridovich, 1978).

Bisphenol A treatment caused significant decrease in protein content in cauda epididymis of mice (Figure 3). Proteins are major targets of free radicals (Barnes *et al.,* 2008). Oxidative modifications such as breakdown of peptide bonds can alter protein structures which are related to reduction of protein by elevated oxidative free radicals (Stadman, 2001). Oral exposure of BPA for 45 days caused significant, dose-dependent decrease in sialic acid content in the cauda epididymis of mice. The altered sialic acid content might affect structural integrity of the acrosomal membrane of sperm (Agarwal *et al.,* 2016). This could also affect sperm morphology. It is also concerned with changing the membrane surface of maturing spermatozoa including stabilization of the acrosome and its membrane during sperm maturation and the coating of spermatozoa with certain antigens, which play an important role in the development of fertilizing capacity of spermatozoa (Levinsky *et al.,* 1983; La Spina *et al.,* 2017). According to Samova *et al.* (2016), BPA exposure causes the morphological abnormality in mice spermatozoa. El-Missiry reported that ascorbic acid had a protective effect against oxidative damage by free radicals. Reduction in the content of ascorbic acid should relate to the reduction in fertility (Wright *et al.,* 2014). Histopathological alteration was seen in different doses of BPA administration. Similar degenerative changes in histopathology were also observed by Chitra *et al.* (2003).

Conclusion

The present investigation revealed that oral administration of BPA is toxic in a way that induces oxidative stress by generating free radicals as well as reducing the activities of enzymatic antioxidants. Moreover, reduction in metabolism and the contents like protein and sialic acid are also seen. This can be a possible reason for reduction in sperm count and increase in sperm morphological abnormalities and may be the reason for reduction in fertility.

REFERENCES

Agarwal A, Bertolla RP, Samanta L. (2016). Sperm proteomics: potential impact on male infertility treatment. *Expert Rev Proteomics* **13**(3): 285–296.

Agarwal A, Virk G, Ong C, du Plessis SS. (2014). Effect of oxidative stress on male reproduction. *World J Mens Health* **32**(1): 1–17.

Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. (2016). Causes and consequences of oxidative stress in spermatozoa. *Reproduction Fertil Dev* **28**(2): 1–10.

Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN. (2014). Oxidative stress and male reproductive health. *Asian J Androl* **16**(1): 31.

Amaral SS Tavares R, Baptista MI, Sousa M, Silva A, Escada-Rebelo S, Paiva PC. and Ramalho-Santos J. (2016). Mitochondrial Functionality and Chemical Compound Action on Sperm Function. *Curr Med Chem* **23**(31): 3575–3606.

Barnes S, Shonsey EM, Eliuk SM, Stella D, Barrett K, Srivastava OP, Kim H, Renfrow MB. (2008). High-resolution mass spectrometry analysis of protein oxidations and resultant loss of function. *Biochem Soc Trans* **36**(5): 1037–1044.

Beatty CH, Basinger GM, Dully CC, Bocek RM. (1966). Comparison of red and white voluntary skeletal muscles of several species of primates. *Journal of Histochemistry & Cytochemistry.* **14**(8): 590–600.

Bindhumol V, Chitra KC, Mathur PP. (2003). Bisphenol A induces reactive oxygen species generation in the liver of male rats. *Toxicology.* **188**(2): 117–124.

Carwile JL, Luu HT, Bassett LS, Driscoll DA, Yuan C, Chang JY, Ye X, Calafat AM, Michels KB. (2009). Polycarbonate bottle use and urinary bisphenol A concentrations. *Environ Health Perspect* **117**(9): 1368.

Chen M, Xu B, Ji W, Qiao S, Hu N, Hu Y, Wu W, Qiu L, Zhang R, Wang Y, Wang S. (2012). Bisphenol A alters n-6 fatty acid composition and decreases antioxidant enzyme levels in rat testes: a LC-QTOF-based metabolomics study. *PloS one.* **7**(9): e44754.

Chitra KC, Rao KR, Mathur PP. (2003). Effect of bisphenol A and co-administration of bisphenol A and vitamin C on epididymis of adult rats: a histological and biochemical study. *Asian J Androl* **5**(3): 203–208.

Chitra KC, Sujatha R, Latchoumycandane C, Mathur PP. (2001). Effect of lindane on antioxidant enzymes in epididymis and epididymal sperm of adult rats. *Asian J Androl* **3**(3): 205–8.

Clermont Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* **52**(1): 198–236.

Collins AJ, Lewis DA. (1971). Lysosomal enzyme levels in the blood of arthritic rats. *Biochem Pharmacol* **20**(1): 251–253.

Cornwall GA. (2009). New insights into epididymal biology and function. *Hum Reprod Update* **15**(2): 213–227.

Delclos KB, Camacho L, Lewis SM, Vanlandingham MM, Latendresse JR, Olson GR, Davis KJ, Patton RE, da Costa GG, Woodling KA, Bryant MS. (2014). Toxicity evaluation of bisphenol A administered by gavage to Sprague-Dawley rats from gestation day 6 through postnatal day 90. *Toxicol Sci* **139**(1): 174–97.

El-Missiry MA, Othman AI, Al-Abdan MA, El-Sayed AA. (2014). Melatonin ameliorates oxidative stress, modulates death receptor pathway proteins, and protects the rat cerebrum against bisphenol-A-induced apoptosis. *J Neurol Sci* **347**(1): 251–256.

Elswefy SES, Abdallah FR, Atteia HH, Wahba AS, Hasan RA. (2016). Inflammation, oxidative stress and apoptosis cascade implications in bisphenol A-induced liver fibrosis in male rats. *Int J Exp Pathol* **97**(5): 369–379.

Fridovich I. (1978). The biology of oxygen radicals. *Science* **201**(4359): 875–880.

Gabrielsen JS, Tanrikut C. (2016). Chronic exposures and male fertility: the impacts of environment, diet, and drug use on spermatogenesis. *Andrology* **4**(4): 648–661.

Gómez-Sintes R, Ledesma MD, Boya P. (2016). Lysosomal cell death mechanisms in aging. *Ageing Res Rev* **32**: 150–168.

Hassan ZK, Elobeid MA, Virk P, Omer SA, ElAmin M, Daghestani MH, AlOlayan EM. (2012). Bisphenol A induces hepatotoxicity through oxidative stress in rat model. *Oxid Med Cell Longev* **2012**: 194829.

Hess RA, de Franca LR. (2009). Spermatogenesis and cycle of the seminiferous epithelium. In *Molecular Mechanisms in Spermatogenesis*. Springer New York. 1–15

Iacobazzi V, Infantino V. (2014). Citrate–new functions for an old metabolite. *Biol Chem* **395**(4): 387–399.

Jourdian GW, Dean L, Roseman S. (1971). The sialic acids XI. A periodate-resorcinol method for the quantitative estimation of free sialic acids and their glycosides. *J Biol Chem* **246**(2): 430–435.

Kabuto H, Hasuike S, Minagawa N, Shishibori T. (2003). Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. *Environ Res* **93**(1): 31–35.

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Kakkar P, Das B, Viswanathan PN. (1984). A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* **21**: 130–132.

Kimura T, Kimura N, Totsukawa K. (2007). Effect of Compound Exposure to Bisphenol A and Nonylphenol on the Development and Fertility of Fetal Mice. *J Mamm Ova Res* **24**(1): 35–41.

La Spina FA, Stival C, Krapf D, Buffone MG. (2017). Molecular and cellular aspects of mammalian sperm acrosomal exocytosis. In: *Animal Models and Human Reproduction*, Schatten H, Constantinescu GM, eds., Wiley-Blackwell, pp. 409–426.

Lanning LL, Creasy DM, Chapin RE, Mann PC, Barlow NJ, Regan KS, Goodman DG. (2002). Recommended approaches for the evaluation of testicular and epididymal toxicity. *Toxicol Pathol* **30**(4): 507–520.

Lei XG, Zhu JH, Cheng WH, Bao Y, Ho YS, Reddi AR, Holmgren A, Arnér ES. (2016). Paradoxical roles of antioxidant enzymes: basic mechanisms and health implications. *Physiol Rev* **96**(1): 307–364.

Levinsky H, Singer R, Barnet M, Sagiv M, Allalouf D. (1983). Sialic acid content of human spermatozoa and seminal plasma in relation to sperm counts. *Arch Androl* **10**(1): 45–46.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**(1): 265–275.

Luck H. (1963). A spectrophotometric method for the estimation of catalase. In *Methods of Enzymatic Analysis*, Bergmeyer HU (ed.), Academic Press, New York, NY, USA, pp. 886–887.

Ohkawa H, Ohishi N, Yagi K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95**(2): 351–358.

Paglia DE, Valentine WN. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Transl Res* **70**(1): 158–169.

Parker RM. (2006). Testing for reproductive toxicity. In: *Developmental and Reproductive Toxicology*. Hood RD (ed.), Taylor & Francis, Boca Raton, pp. 425–488.

Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. (2012). The role of mitochondria in energy production for human sperm motility. *Int J Androl* **35**(2): 109–124.

Prescott C, Bottle SE. (2016). Biological Relevance of Free Radicals and Nitroxides. *Cell Biochem Biophys* **75**(2): 227–240

Putilina PE, Eschanko ND. (1969). Activity of some Kreb's cycle dehydrogenases in the brain, liver and kidneys. Yestln. Leningrad . *Univ Ser Biol* **24:** 112–116.

Qiu LL, Wang X, Zhang XH, Zhang Z, Gu J, Liu L, Wang Y, Wang X, Wang SL. (2013). Decreased androgen receptor expression may contribute to spermatogenesis failure in rats exposed to low concentration of bisphenol A. *Toxicol Lett* **219**(2): 116–124.

Quinn PJ, White IG 1968 Distribution of adenosinetriphosphatase activity in ram and bull spermatozoa. *J Reprod Fertil* **15**(3): 449–452.

Ramalho-Santos J, Varum S, Amaral S, Mota PC, Sousa AP, Amaral A. (2009). Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Human Reprod Update*. **15**(5): 553–572.

Ranjit N, Siefert K, Padmanabhan V. (2010). Bisphenol-A and disparities in birth outcomes: a review and directions for future research. *J Perinatol* **30**(1): 2–9.

Robaire B, Hermo L. (1988). Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: *The Physiology of Reproduction*, Knobil E. *et al*. (eds.), Raven Press, New York, NY, pp. 999–1080.

Rochester JR. (2013). Bisphenol A and human health: a review of the literature. *Reprod Toxicol* **42**: 132–155.

Samova S, Doctor H, Verma RJ. (2016). Spermatotoxic effect of bisphenol a and its amelioration using quercetin. *World J Pharm Pharm Sci* **5**(5): 1161–1175.

Sangai NP, Verma RJ. (2012). Quercetin ameliorates bisphenol A-induced toxicity in mice. *Acta Pol Pharm Drug Res* **69**(3): 557–563.

Serre V & Robaire B. (1998). Segment-specific morphological changes in aging brown Norway rat epididymis. *Biol Reprod* **58**(2): 497–513.

Sigma Technical Bulletin No. 104, Sigma Chemical Co., 3500, Dekoib St. Louis 18, MO, USA, 2001.

Stadman ER. (2001). Protein oxidation in aging and age-related diseases. *Ann N Y Acad Sci* **928**(1): 22–38.

Staples CA, Dome PB, Klecka GM, Oblock ST, Harris LR. (1998). A review of the environmental fate, effects, and exposures of bisphenol A. *Chemosphere*. **36**(10): 2149–2173.

Takahashi O, Oishi S. (2001). Testicular toxicity of dietary 2, 2-bis (4-hydroxyphenyl) propane (bisphenol A) in F344 rats. *Arch Toxicol* **75**(1): 42–51.

Vandenberg LN, Chahoud I, Heindel JJ, Padmanabhan V, Paumgartten FJ, Schoenfelder G. (2010). Urinary, circulating, and tissue biomonitoring studies indicate widespread exposure to bisphenol A. *Environ Health Perspect* **118**(8): 1055–1070.

Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. (2007). Human exposure to bisphenol A (BPA). *Reprod Toxicol* **24**(2): 139–177.

Verma RJ & Sangai NP. (2009). The ameliorative effect of black tea extract and quercetin on bisphenol A-induced cytotoxicity. *Acta Pol Pharm*. **66**(1): 41–44.

Vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV. (1998). A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicol Ind Health* **14**(1–2): 239–260.

Wetherill YB, Akingbemi BT, Kanno J, McLachlan JA, Nadal A, Sonnenschein C, Watson CS, Zoeller RT, Belcher SM. (2007). *In vitro* molecular mechanisms of bisphenol A action. *Reprod Toxicol* **24**(2): 178–198.

Wright C, Milne S, Leeson H. (2014). Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. *Reprod Biomed Online* **28**(6): 684–703.

Xia W, Jiang Y, Li Y, Wan Y, Liu J, Ma Y, Mao Z, Chang H, Li G, Xu B, Chen X. (2014). Early-life exposure to bisphenol a induces liver injury in rats involvement of mitochondria-mediated apoptosis. *PloS one*. **9**(2): p.e90443.

Zeisel SH. (2012). Dietary choline deficiency causes DNA strand breaks and alters epigenetic marks on DNA and histones. *Mutat Res* **733**(1): 34–38.

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ORIGINAL ARTICLE

Beneficial effect of 6 weeks lasting handling of adult rats on spatial memory in experimental model of neurodegeneration

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ABSTRACT

Handling is a form of experience which can result in physiological changes depending on the period of postnatal age when performed. There is a lot of evidence about the positive effect of neonatal handling, but a lack dealing with handling of adult rats. Behavioral changes and memory deficits are present in dementia-like disorders. In the present work, we tested whether 6 weeks lasting handling of young adult rats could revert memory impairment induced by trimethyltin (TMT) (7.5 mg/kg, intraperitoneally). Testing rats in Morris water maze revealed significant effect of TMT as well significant effect of handling. We observed improvement of spatial memory also between healthy, non-degenerated rats as well as degenerated rats, represented by shorter latency onto the platform. In our paper, we report beneficial effect of handling on spatial memory that is in compliance with published works about beneficial effect of cognitive therapy and training in patients with early stage of Alzheimer´s disease and dementia.

KEY WORDS: trimethyltin; handling; memory; neurodegeneration

Introduction

Life experiences and environmental factors can modulate animal physiology, behavior and memory (Del Arco *et al.*, 2007; Levine, 2001; Winterfeld, 1998). In 2003 Frick and Fernandez demonstrated beneficial effect of enrichment on spatial memory in adult female mice. Another intervention, handling, was broadly investigated in neonates. In adult and old rats, early postnatal handling has been shown to reduce anxiety, decrease levels of prolactin and corticosterone following stress, prevent age-related loss of hippocampal CA1 and CA3 pyramidal cells and deterioration of working memory and recognition memory (Boufleur *et al.*, 2013; Fenoglio *et al.*, 2005; Ferré *et al.*, 1995; Meaney *et al.*, 1988; Meerlo *et al.*, 1999; Stamatakis *et al.*, 2008; Valée *et al.*, 1999; Viau *et al.*, 1993). The above mentioned neonatal handling mediated memory improvement was accompanied by increase in dendritic length and dendritic spine density in the cortex (Richards *et al.*, 2012). Positive effect of early postnatal handling has been shown on prenatal stress, alcohol exposure and malnutrition in experimental rats (Raineki *et al.*, 2014). Much less is known about the consequence of handling of adult rats (Costa *et al.*, 2012; Deutsch-Feldman *et al.*, 2015).

In our study, we focused on the effect of late postnatal handling on spatial memory in experimental model of dementia induced by trimethyltin (TMT). TMT has a harming effect on the hippocampus that together with memory damage makes TMT a useful tool for studying perspective neuroprotective drugs (Geloso *et al.*, 2011). With the perspectivity of stannin, a key protein involved in TMT sensitivity, TMT is responsible for disruption of calcium homeostasis, production of reactive oxygen species, increment in levels of malondialdehyde, astrocyte activation and reactive gliosis, as well as the spread of neuronal apoptosis leading to functional deterioration of synaptic transmission (Corvino *et al.*, 2011; Gasparova *et al.*, 2012; Kaur *et al.*, 2013; Piacentini *et al.*, 2008; Thompson *et al.*, 1996). It is learning and cognition decline that is a typical hallmark of dementia (Gutiérrez-Rexach & Schatz, 2016; Popa-Wagner *et al.*, 2015). In patients with

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cognitive impairment tactile stimulation and cognitive exercise improved memory (Gates *et al.,* 2011; Scherder *et al.,* 1995).

Our hypothesis to test the effect of handling on neurodegeneration-induced rats was based on our previous observation when 28 days lasting oral administration of *aqua pro injectione* (vehiculum) to rats showed better achievement in Morris water maze test compared to our control groups of rats in experiments without such regular manipulation with animals. We wanted to verify whether handling could be beneficial not only for unaffected control rats but even for neurodegeneration induced rats. We decided to test rat spatial memory in Morris water maze, a behavioral test best suitable for testing spatial memory and learning (Vorhees & Williams, 2006) on the model of TMT-induced neurodegeneration with aspect of handling. In the current study we report beneficial effect of late handling on learning and spatial memory in intact and degenerated adult rats.

Materials and methods

Animals

Male Wistar rats (n=32), 8 weeks old, weighing 197±1.3 g at the beginning of the 8-week lasting experiment came from the breeding station Dobra Voda of the Institute of Experimental Pharmacology and Toxicology (Slovak Republic, reg. No. SK CH 24011). All procedures with animals were performed in compliance with the principles of laboratory animal care issued by EU Directive 2010/63/EU for animal experiments, proved and controlled by the State Veterinary and Food Administration of Slovakia and the Ethical Committee of the Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences. The rats were housed in plastic cages (4 rats per cage) with pelletful food (KKZ-P/M) and water *ad libitum*.

Experimental procedure

After 7-day adaptation, the rats were randomly allocated into 4 groups (n=8/group):

- Control handled group (C-H),
- Control non-handled group (C-NH),
- TMT handled group (TMT-H),
- TMT non-handled group (TMT-NH).

Control rats received a single i.p. dose of saline (with 0.1% DMSO). TMT rats were affected by a single i.p. dose of TMT chloride (7.5 mg/kg; dissolved in 0.1% DMSO; Sigma-Aldrich, USA) in the volume of 0.2 ml/100 g of rat weight. Four weeks prior to the TMT or vehicle administration, handling was performed on the C-H and TMT-H groups. Altogether handling lasted for 6 weeks (7 days per week), including 2 weeks after TMT/vehicle administration, and ended one week before the Morris water maze test. Handling consisted of tactile stimulation of rats, 10 minutes per cage/day. The rats were also allowed to enter the nearest table next to their cage during handling. Non-handled groups of rats were left undisturbed in the next room.

Morris water maze

The testing in Morris water maze was performed with the hidden platform positioned on the same place during five subsequent days without probe trial on last day according to Gasparova *et al.* (2014). Briefly, the rats were tested in the range of the 21st till the 25th day (beginning at 7 a.m.) after TMT/or saline administration. The platform was hidden 0.5 cm under the water (23 °C) surface of the pool with a diameter of 180 cm. Each rat was placed in succesive steps into each of the 4 quadrants of the pool every day. The rat had 60 seconds to find the platform. After finding the platform, the animal was left on the platform for 20 seconds. After completion of the 4th quadrant, each animal was carefully dried and placed under a lamp. The data were collected by a camera located above the pool and connected to the computer with the ANY-maze videotracking software (Stoelting Europe, Ireland).

Statistics

Values are means ± SEM. Data obtained from Morris water maze were analyzed using Statistica 7.0 software, Fisher´s least significant difference test. Body mass gain was analyzed using two factorial ANOVA for independent samples.

Results

Six weeks lasting handling resulted in significant reduction of body weight gain in both handled groups compared to respective non-handled groups. Body weight gain of handled TMT-treated group did not differ from handled saline-treated group (Figure 1). The observed body weight gain reduction was the effect of handling in the mentioned corresponding groups ($F(1,28)=20.93$; $p<0.001$).

Percentual expression of body weight gain of individual groups calculated as a difference between the body weight on the 1st day of handling and after 6 weeks of handling, at the end of the experiment. C-NH group, C-H group, TMT-NH group, TMT-H group. Data are expressed as mean ± SEM, $^{*}p<0.05$.

In the Morris water maze test, data revealed significant positive effect of handling ($F(1,28)=10.05$; $p<0.01$) (Figure 2A) and negative effect of treatment with TMT ($F(1,28)=15.23$; $p<0.001$) (Figure 2B) on escape latency to the platform.

The effect of handling is expressed as cumulative mean value of escape latency to the platform from both non-handled (NH) *vs.* handled (H) groups (A) and the effect of treatment is expressed as cumulative mean value from both non-treated (C) *vs.* both TMT-treated groups (TMT) (B). Data are expressed as mean ± SEM, $^{**}p<0.01$, $^{***}p<0.001$.

The trend of improved spatial memory of handled groups is shown in Figure 3A. Note that the escape latency for TMT-H group is similar to the C-NH group. Total mean escape latency during 5 consecutive days revealed significant amelioration of spatial memory between C-NH and C-H and TMT-NH and TMT-H (Figure 3B).

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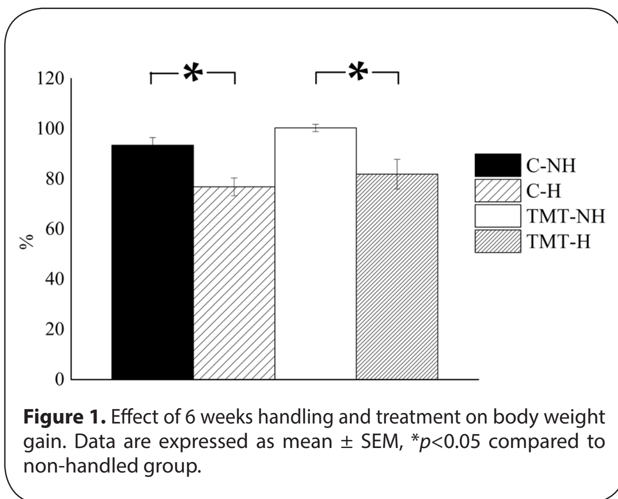

Figure 1. Effect of 6 weeks handling and treatment on body weight gain. Data are expressed as mean ± SEM, *$p<0.05$ compared to non-handled group.

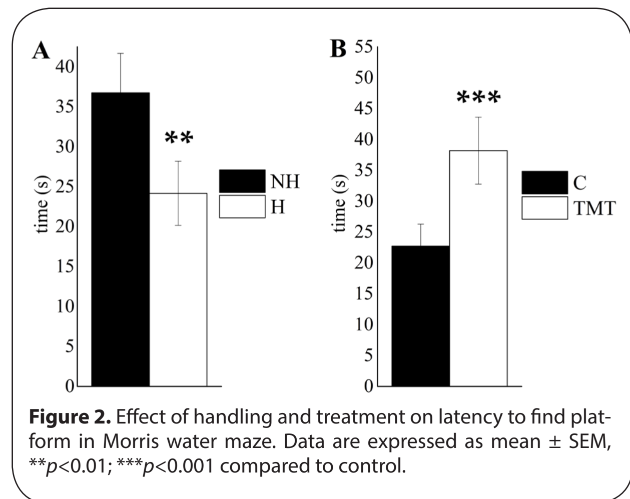

Figure 2. Effect of handling and treatment on latency to find platform in Morris water maze. Data are expressed as mean ± SEM, **$p<0.01$; ***$p<0.001$ compared to control.

Discussion

Diagnostical guidelines characterize Alzheimer dementia (AD) as a continuum dividing it to preclinical stage without symptoms, mild cognitive impairment (MCI) and finally AD (Albert *et al.,* 2011). Garrett and Valle (2014) doubt about this division because of the difficulty in diagnosis, non-uniformity and the confession that memory is malleable, as shown by cognitive training programs in humans (Garrett & Valle, 2014; Kurz *et al.,* 2009). We rate TMT-induced neurodegeneration as a mild cognitive impairment with no neurofibrillary tangles and senile plaques.

In the rat, we show a modulatory effect of long-lasting postnatal handling on spatial memory and learning. Beyond the positive effect of handling on neurodegeneration-induced rats, we also report improvement of spatial memory in control, healthy rats compared to control non-handled group. Costa *et al.* (2012) showed anxiety reducing effect of handling with improved memory tested in elevated plus-maze test.

Beneficial effect of cognitive training was already observed in patients with MCI and AD (Belleville *et al.,* 2006) and here we demonstrate the beneficial effect of postnatal handling on memory of adult animals with induced neurodegeneration. Along the effect on memory, we also observed significant decrease in body weight of handled rats. On the contrary, Panagiotaropoulos *et al.* (2004) and Vallée *et al.* (1996) observed neonatal handling mediated increase in body weight of rats as well as increased food intake in adulthood. Another study using young adult rats revealed no effect of handling of young rats on body weight (Deutsch-Feldman *et al.,* 2015). The reason of discrepancies may origin from the period of development when handling was performed, as well as the duration of handling, that was in our experiment much longer than in the studies mentioned.

In conclusion, we report ameliorated spatial memory in healthy non-treated rats and rats with TMT-induced neurodegeneration when handled for 6 weeks. Because of lack of studies about the effect of handling in adulthood, the explanation of the precise mechanism is still shrouded in mystery. There is a suggestion that the effect of the procedure used in the present experiment and positive effect of cognitive training and tactile stimulation in patients with MCI and AD-like dementia can be based on similar mechanisms. Further investigation is necessary for complete description of this phenomenon.

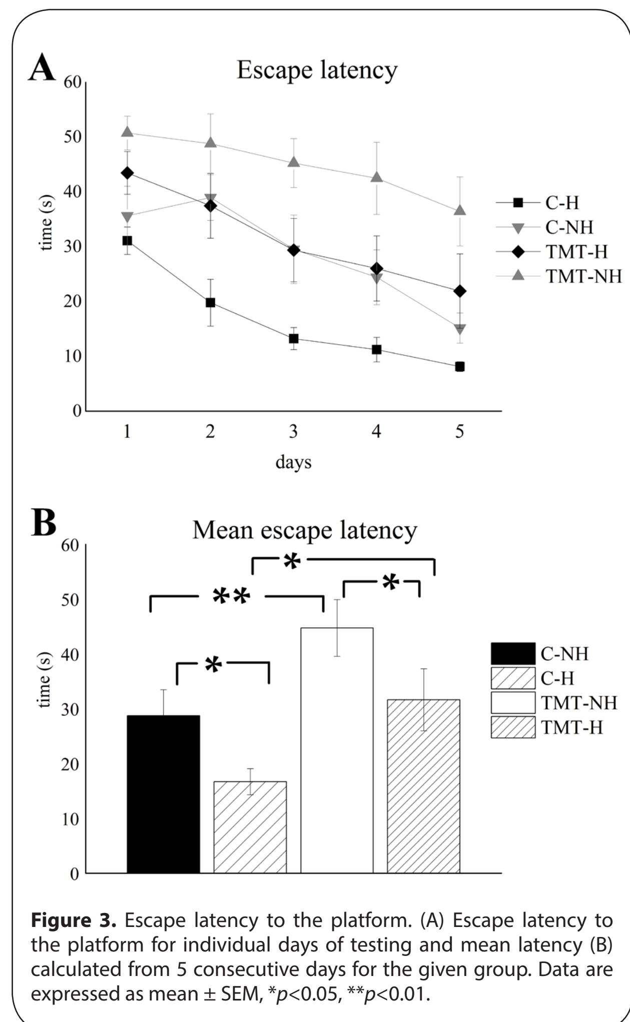

Figure 3. Escape latency to the platform. (A) Escape latency to the platform for individual days of testing and mean latency (B) calculated from 5 consecutive days for the given group. Data are expressed as mean ± SEM, *$p<0.05$, **$p<0.01$.

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REFERENCES

Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM, Jagust WJ, Petersen RC, Snyder PJ, Carrillo MC, Thies B, Phelps CH. (2011). The diagnosis of mild cognitive impairment due to Alzheimer' s disease: recommendations from the National Institute on Aging- Alzheimer' s Association workgroups on diagnostic guidelines for Alzheimer' s disease. *Alzheimers Dement* **7**: 270–279.

Belleville S, Gilbert B, Fontaine F, Gagnon L, Ménard E, Gauthier S. (2006). Improvement of episodic memory in persons with mild cognitive impairment and healthy older adults: evidence from a cognitive intervention program. *Dement Geriatr Cogn Disord* **22**: 486–499.

Boufleur N, Antoniazzi CT, Pase CS, Benvegnú DM, Dias VT, Segat HJ, Roversi K, Rovesi K, Nora MD, Koakoskia G, Rosa JG, Barcellos LJ, Bürger ME. (2013). Neonatal handling prevents anxiety-like symptoms in rats exposed to chronic mild stress: behavioral and oxidative parameters. *Stress* **16**: 321–330.

Corvino V, Marchese E, Zarkovic N, Zarkovic K, Cindric M, Waeg G, Michetti F, Geloso MC. (2011). Distribution and time-course of 4-hydroxynonenal, heat shock protein 110/105 family members and cyclooxygenase-2 expression in the hippocampus of rat during trimethyltin-induced neurodegeneration. *Neurochem Res* **36**: 1490–1500.

Costa R, Tamascia ML, Nogueira MD, Casarini DE, Marcondes FK. (2012). Handling of adolescent rats improves learning and memory and decreases anxiety. *J Am Assoc Lab Anim Sci* **51**: 548–553.

Del Arco A, Segovia G, Garrido P, de Blas M, Mora F. (2007). Stress, prefrontal cortex and environmental encrichment: studies on dopamine and acetylcholine release and working memory performance in rats. *Behav Brain Res* **176**: 267–273.

Deutsch-Feldman M, Picetti R, Seip-Cammack K, Zhou Y, Kreek MJ. (2015). Effects of handling and vehicle injections on adrenocorticotropic and corticosterone concentrations in Sprague-Dawley compared with Lewis rats. J Am Assoc Lab Sci **54**: 35–39.

Fenoglio KA, Brunson KL, Avishai-Eliner S, Stone BA, Kapadia BJ, Baram TZ. (2005). Enduring, handling-evoked enhancement of hippocampal memory function and glucocorticoid receptor expression involves activation of the corticotropin-releasing factor type 1 receptor. *Endocrinology* **146**: 4090–4096.

Ferré P, Núñez JF, García E, Tobeña A, Escorihuela RM, Fernández-Teruel A. (1995). Postnatal handling reduces anxiety as measured by emotionality rating and hyponeophagia test sin female rats. *Pharmacol Biochem Behav* **51**: 199–203.

Frick KM, Fernandez SM. (2003). Enrichment enhances spatial memory and increases synaptophysin levels in aged female mice. *Neurobiol Aging* **24**: 615–626.

Garrett MD, Valle R. (2016). A methodological critique of the National Institute of Aging and Alzheimer´s Association Guidelines for Alzheimer´s disease, dementia, and mild cognitive impairments. *Dementia* **15**: 239–254.

Gasparova Z, Janega P, Stara V, Ujhazy E. (2012). Early and late stage of neurodegeneration induced by trimethyltin in hippocampus and cortex of male Wistar rats. *Neuro Endocrinol Lett* **33**: 689–696.

Gasparova Z, Stara V, Janega P, Navarova J, Sedlackova N, Mach M, Ujhazy E. (2014). Pyridoindole antioxidant-induced preservation of rat hippocampal pyramidal cell nubmer linked with reduction of oxidative stress yet without influence on cognitive deterioration in Alzheimer-like neurodegeneration. *Neuro Endocrinol Lett* **35**: 454–462.

Gates NJ, Sachdev PS, Fiatarone Singh MA, Valenzuela M. (2011). Cognitive and memory training in adults at risk of dementia: a systematic review. *BMC Geriatr* **11**: 55.

Geloso MC, Corvino V, Michetti F. (2011). Trimethyltin-induced hippocampal degeneration as a tool to investigate neurodegenerative process. *Neurochem Int* **58**: 729–739.

Gutiérrez-Rexach J, Schatz S. (2016). Cognitive impairment and pragmatics. *Springerplus* **5**:127.

Kaur S, Chhabra R, Nehru B. (2013). Ginkgo biloba extract attenuates hippocampal neuronal loss and cognitive dysfunction resulting from trimethyltin in mice. *Phytomedicine* **20**: 178–86.

Kurz A, Pohl C, Ramsenthaler M, Sorg C. (2009). Cognitive rehabilitation in patients with mild cognitive impairment. *Int J Geriatr Psychiatry* **24**: 163–168.

Levine S. (2001). Primary social relationships influence the development of the hypothalamic-pituitary-adrenal axis in the rat. *Physiol Behav* **73**: 255–60.

Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM. (1988). Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science* **239**: 766–768.

Meerlo P, Horvath KM, Nagy GM, Bohus B, Koolhaas JM. (1999). The influence of postnatal handling on adult neuroendocrine and behavioural stress reactivity. *J Neuroendocrinol* **11**: 925–933.

Panagiotaropoulos T, Papaioannou A, Pondiki S, Prokopiou A, Stylianopoulou F, Gerozissis K. (2004). Effect of neonatal handling and sex on basal and chronic stress-induced corticosterone and leptin secretion. *Neuroendocrinology* **79**: 109–118.

Piacentini R, Gangitano C, Ceccariglia S, Del Fà A, Azzena GB, Michetti F, Grassi C. (2008). Dysregulation of intracellular calcium homeostasis is responsible for neuronal death in an experimental model of selective hippocampal degeneration induced by trimethyltin. *J Neurochem* **105**: 2109–2121.

Popa-Wagner A, Buga AM, Popescu B, Muresanu D. (2015). Vascular cognitive impairment, dementia, aging and energy demand. A vivious cycle. *J Neural Transm* **122**: S47–54.

Raineki C, Lucion AB, Weinberg J. (2014). Neonatal handling: an overview of the possitive and negative effects. *Dev Psychobiol* **56**: 1613–1625.

Richards S, Mychasiuk R, Kolb B, Gibb R. (2012). Tactile stimulation during development alters behaviour and neuroanatomical organization of normal rats. *Behav Brain Res* **231**: 86–91.

Scherder E, Bouma A, Steen L. (1995). The effects of peripheral tactile stimulation on memory in patients with probable Alzheimer´s disease. *Am J Alzheimer* Dis **10**: 15–21.

Stamatakis A, Pondiki S, Kitraki E, Diamantopoulou A, Panagiotaropoulos T, Raftogianni A, Stylianopoulou F. (2008). Effetc of neonatal handling on adult spatial learning and memory following acute stress. *Stress*, **11**: 148–159.

Thompson TA, Lewis JM, Dejneka NS, Severs WB, Polavarapu R, Billingsley ML. (1996). Induction of apoptosis by organotin compounds in vitro: neuronal protection with antisense oligonucleotides directed against stannin. *J Pharmacol Exp Ther* **276**: 1201–1216.

Vallée M, Mayo W, Maccari S, Le Moal M, Simon H. (1996). Long-term effects of prenatal stress and handling on metabolic parameters: relationship to corticosterone secretion response. *Brain Res* **712**: 287–292.

Vallée M, MacCari S, Dellu F, Simon H, Le Moal M, Mayo W. (1999). Long-term effects of prenatal stress and postnatal handling on age-related glucocorticoid secretion and cognitive performance: a longitudinal study in the rat. *Eur J Neurosci* **11**: 2906–2916.

Viau V, Sharma S, Plotsky PM, Meaney MJ. (1993). Increased plasma ACTH responses to stress in nonhandled compared with handled rats require basal levels of corticosterone and are associated with increased levels of ACTH secretagogues in the median eminence. *J Neurosci* **13**: 1097–1105.

Vorhees CV, Williams MT. (2006). Morris water maze: procedure for assessing spatial and relate forms of learning and memory. *Nat Protoc* **1**: 484–458.

Winterfeld KT, Teuchert-Noodt G, Dawirs RR. (1998). Social environment alters both ontogeny of dopamine innervation of the medial prefrontal cortex and maturation of working memory in gerbils (*Meriones unguiculatus*). *J Neurosci Res* **52**: 201–209.

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ORIGINAL ARTICLE

Sub-chronic oral toxicity assessment (90 days) of ethanolic fraction of leaves of *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. in rodents: A lesser known ethnomedicinal plant from the Cholanaickan tribal community, India

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ABSTRACT

The objective of the present study was to evaluate the safety of long term consumption of ethanolic fraction of *Neurocalyx calycinus* leaves (NCEF) in rodents. The NCEF was subjected to detect the presence of various phytoconstituents. In acute oral toxicity study, graded doses of NCEF was administered in mice and were observed up to 14 days. In sub-chronic oral toxicity study, NCEF was administered to Wistar rats at doses of 50, 500 and 1000 mg/kg b.w. per day for 90 days and after that, observed up to 28 days. NCEF showed the presence of alkaloids, steroids, phenolics and glycosides. In acute toxicity study, there was no mortality and no behavioural signs of toxicity at the highest dose level (6400 mg/kg b.w.). In sub-chronic oral toxicity study, there were no significant difference observed in the consumption of food and water, body weight and relative organ weights. Haematological, serum biochemical, hepatic oxidative stress marker analysis and urine analysis revealed the non-adverse effects of prolonged oral consumption of NCEF. The histopathologic examination did not show any differences in vital organs. Based on our findings, NCEF, at dosage levels up to 1000 mg/kg b.w., is non-toxic and safe for long term oral consumption.

KEY WORDS: *Neurocalyx calycinus*; sub-chronic toxicity; histopathology

ABBREVIATIONS

ASL: above sea level; **ABR:** Agasthyamalai Biosphere Reserve; **NCEF:** ethanolic fraction of leaves of *Neurocalyx calycinus*; **b.w.:** body weight; **v/v:** volume/volume; **w/v:** weight/volume; **EDTA:** ethylenediaminetetraacetic acid; **WBC:** white blood cell count; **RBC:** red blood cell count; **HGB:** hemoglobin; **HCT:** hematocrit; **MCV:** mean corpuscular volume; **MCH:** mean corpuscular hemoglobin; **MCHC:** mean corpuscular hemoglobin concentration; **RDW:** red blood cell distribution width; **PLT:** platelet count; **MPV:** mean platelet volume; **PDW:** platelet distribution width; **PCT:** plateletcrit; **LYM:** lymphocyte count; **MONO:** monocyte count; **GRAN:** granulocyte count; **ALT:** alanine transaminase; **AST:** aspartate transaminase; **ALP:** alkaline phosphatase; **GGT:** γ-glutamyltransferase; **LDH:** L-lactate dehydrogenase; **TSP:** total serum protein; **ALB:** albumin; **TBIL:** total bilirubin; **GLU:** glucose; **CK:** creatine kinase; **CRE:** creatinine; **BUN:** urea nitrogen; **UA:** uric acid; **TG:** serum triglyceride; **TC:** total cholesterol; **Ca^{2+}:** calcium; **Na^{+}:** sodium; **K^{+}:** potassium; **Cl^{-}:** chlorine; **PBS:** phosphate buffer saline; **THP:** total hepatic protein; **BSA:** bovine serum albumin; **CAT:** catalase; **GSH:** reduced glutathione; **SOD:** superoxide dismutase; **MDA:** malondialdehyde; **GPx:** glutathione peroxidase; **GLU:** glucose; **BIL:** bilirubin; **KET:** ketone; **SG:** specific gravity; **BLO:** blood; **PRO:** protein; **URO:** urobilinogen; **NIT:** nitrite; **LEU:** leukocytes; **DTNB:** (5,5′-dithio-bis-[2-nitrobenzoic acid]); **NBT:** nitro blue tetrazolium; **NADH:** nicotinamide adenine dinucleotide; **TBA:** thiobarbituric acid; **H_2O_2:** hydrogen peroxide.

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Introduction

The diverse ecological conditions in India make it a treasure house of biodiversity, covering about 8% of global biodiversity. The forests of India are estimated to harbour 90% of India's medicinal plant diversity in a wide range

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of forest types (Aneesh *et al*., 2009). With its rich ethnic diversity and strong traditional knowledge in herbal medicine, India has been using herbal drugs for thousands of years. Due to high prices and harmful side effects of synthetic drugs, people rely more on herbal drugs as evidenced by its rapidly growing trends in international and national markets through exploration of ethnopharmacology and traditional medicine (Fabricant & Farnsworth, 2001). The modern drug discovery screening techniques, mainly based on traditional knowledge, have given clues to the development of valuable drugs (Hashmi & Singh, 2003). Most of the traditional knowledge about medicinal plants was in the form of oral knowledge that has been eroded or distorted due to the persistent invasions and cultural adaptations. Locally available medicinal plants are primary raw material for the development of traditional drug formulations.

Neurocalyx Hook is an endemic taxon in the family Rubiaceae, mainly distributed in South Western Ghats of India and Sri Lanka (Bremer, 1979). *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. is a wild ornamental herbaceous plant classified under the tribe Ophiorrhizeae (Bremer, 1987; Viswanathan *et al*., 2005; Takhtajan, 2009). It grows up to 20 inches and is dispersed on rocky crevices at the banks of streams in the tropical wet evergreen forests at higher altitudes (1200–1600 m ASL). Cholanaickans, the last remaining hunter-gatherer tribes of South India, are the most primitive and vanishing native tribal communities, living in caves called Alas spread in the Karulai and Chungathara forest ranges in Nilambur, Malappuram district of Kerala, India (Menon, 1996; Mathur, 2013). Cholanaickans use the fresh leaves of *N. calycinus*, locally known as 'Pachachedi', prepared in the form of paste and applied externally to arrest bleeding due to bear bites and to heal fresh wounds, inflammation and pain. Decoction of the leaves (30 mL) is administered orally thrice a day for one week to one month, depending upon the conditions of the symptoms (Saradamma *et al*., 1994).

From the review of literature, it was evident that a sparse record is available about the usage, safety parameters and medicinal properties of *N. calycinus*. To develop a novel drug with least side effects and multi therapeutic effect, preclinical studies along with toxicity analysis should be conducted *in vivo* with pertinent animal models. The present work was done to evaluate the acute and subchronic (90 days) toxicity effect of the ethanolic fraction of leaves of *Neurocalyx calycinus* (NCEF) in Wistar rats.

Material and methods

Chemicals

Bovine serum albumin (BSA), Catalase, 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), L-Glutathione, Superoxide Dismutase (SOD), Nitro Blue Tetrazolium (NBT) and Nicotinamide Adenine Dinucleotide (NADH) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Biochemical kits were purchased from Coral Clinical System, Goa, India. All other reagents, chemicals, and solvents were of analytical grade and were purchased from HiMedia Laboratories (India). Phosphate Buffered Saline (PBS) and other reagents were prepared according to protocol.

Plant material

The fresh leaves of *Neurocalyx calycinus* (R. Br. ex Benn.) Rob. (vernacular name: Pachachedi) were collected from the evergreen forest streams of the upper hill of Ezhumadakka (Latitude 8°37'28"N, Longitude 77°12'53"E) in Athirumala at the Agasthyamalai Biosphere Reserve (ABR) region, Thiruvananthapuram, Kerala, India during the month of April 2014. The plant material was taxonomically identified by Dr. A.G. Pandurangan, Plant Taxonomist, Plant Systematics and Evolutionary Science Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Thiruvananthapuram, Kerala, India. A voucher specimen (TBGT 86801 dated 14/12/2015) was certified (JNTBGRI/PS/213/2015) and deposited at the institutional herbarium.

Preparation of the plant extract

The fresh leaves of *Neurocalyx calycinus* were thoroughly washed, segmented, shade dried and powdered (Usha Shriram (India), Noida, UMA 29103448). The powder (100 g) was sequentially extracted with petroleum ether, chloroform and ethanol (96% v/v) in a Soxhlet apparatus for 24 h each. Each time before extracting with the next solvent, marc was air dried at below 35±5 °C. The ethanolic extract was concentrated under reduced pressure at 30±10 mbar in a rotary evaporator at 30–60 °C (Rotavapor R-215, Buchi, Switzerland) to a syrupy consistence (6.28±0.47% w/w) named NCEF, finally dried in a desiccator (Auto Secador 401110, USA) and stored in airtight containers in a refrigerator at 4 °C.

Qualitative phytochemical screening

The NCEF was subjected to qualitative phytochemical tests to detect the presence of various phytoconstituents (Sofowora, 1982; Harborne, 1998) such as carbohydrates (Molisch test, Fehling test, Barfoed test and Benedict test), proteins (Millon test, Biuret test and Ninhydrin test), alkaloids (Mayer test, Wagner test, Hager test and Dragendorff test), glycosides (Borntrager test, Legal test, Keller kiliani test and Kedde test), phenolic compounds (ferric chloride test, gelatin test, lead acetate test, alkaline reagent test and Shinoda test), phytosterols (Libermann-Burchard test and Salkowski test), fixed oils and fats (spot test and saponification test), saponins (foam test), gum and mucilage (alcohol 95% test), volatile oils (steam distillation), anthraquinones (chloroform – 10% ammonia test) and iridoids (Trim-Hill reagent test).

Experimental animals

Adult female and male Swiss Albino mouse (*Mus musculus*), 25±2 g, and Wistar Albino rat (*Rattus norvegicus*), 155±3 g, were obtained from the animal house of JNTBGRI, India. All animals were housed in standard polypropylene cages at controlled temperature (25±2 °C),

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with light conditions (12 h light and dark cycle), room air changes 15±3 times/h and relative humidity (65±5%). The animals were provided with pellet diet (Lipton India Ltd. Mumbai) and water *ad libitum*. The animals were allowed to acclimatize to the new environment for 7 days before starting the experiment. All experimental protocols described in this study were approved by the Institutional Animal Ethics Committee, JNTBGRI and were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), Government of India (B-01/03/2015/EM-EP/05).

Acute oral toxicity study

The acute oral toxic effect of NCEF was studied in Swiss albino mice (Lorke, 1983). Animals (54) of both sexes were randomized into 9 groups of 6 each (Group A to Group I). Before starting the experiment, all groups were fasted for 12 h and weighed. The NCEF was freshly prepared by suspending with 1.5% v/v Tween-80 in distilled water. 0.5 mL of the vehicle and test materials were administered orally *via* gavage No. 16 to Group B to I (NCEF at 50, 100, 200, 400, 800, 1600, 3200, 6400 mg/kg b.w. respectively) and Group A, vehicle control, was given 0.5 mL 1.5% v/v Tween-80 in distilled water. Food and drinking water were provided to the mice approximately an hour after treatment. The animals were observed 30 min after dosing, followed by hourly observation for 8 h and once a day for the next 14 days. Cage side observations were systematically recorded for each animal on day 1, 7, 14 and visual observations for mortality, behavioral pattern, body weight, changes in physical appearance, injury, pain and signs of illness were examined daily.

Sub-chronic oral toxicity study

The sub-chronic oral toxicity studies were conducted according to OECD Guidelines No. 408 (OECD, 1998). Wistar rats (48) were randomized into 4 groups and received 1 mL of the test materials orally once a day consecutively for 90 days. Group A (8 rats/sex) served as vehicle-control and they were given 1.5% v/v Tween-80 in distilled water. Group B and C (4 rats/group/sex) received NCEF at 50 and 500 mg/kg b. w. respectively. Group D (8 rats/sex) received NCEF at 1000 mg/kg b.w. After 90 days of treatment, 4 animals of each sex from group A and D were assigned as satellite groups and kept for further observation after the treatment for a period of 28 days, for any reversibility or persistence of toxic effects.

Mortality checks and behavioral observations

All the animals were observed every day in the morning for mortality and signs of morbidity. Any changes in the skin, fur, subcutaneous swellings, eyes, mucous membranes, excretions, autonomic activity, changes in gait, posture and response to handling as well as bizarre behavior were noted during the entire period of the study.

Food intake and water consumption

The quantities of supplied and remaining food and water were measured daily and average weekly consumption was calculated.

Body weight and relative organ weight

The body weight of all animals was recorded before starting oral administration and continued once a week up to the day of sacrifice. Based on the body weight, the quantities of NCEF were calculated again to ensure administration of fixed dose. The overnight fasted animals were sacrificed on the 91st day and 119th day (satellite group) for dissecting internal organs such as liver, kidneys, lungs, spleen, heart, brain, ovaries and testes. The relative organ weight of each animal was then calculated by the following formula:

Relative organ weight = [absolute organ weight (g)/ body weight of rat on sacrifice day (g)] × 100

Hematological analysis

The blood samples were collected from overnight fasted animals through cardiac puncture on day 91 and day 119. One part was collected into ethylenediaminetetraacetic acid (EDTA)-coated vials and analysis such as white blood cell count (WBC), 2.9 to 15.3×10^3/μL; red blood cell count (RBC), 5.60 to 7.89×10^6/μL; hemoglobin (HGB) concentration, 12 to 15 g/dL; hematocrit (HCT), 36 to 46%; mean corpuscular volume (MCV), 53.0 to 68.8 fL; mean corpuscular hemoglobin (MCH), 16.0 to 23.1 pg; mean corpuscular hemoglobin concentration (MCHC), 30.0 to 34.1 g/dL; red blood cell distribution width (RDW), 11.0 to 15.5%; platelet count (PLT), 100 to 1610×10^3/μL; mean platelet volume (MPV), 3.8 to 6.2 fL; platelet distribution width (PDW); plateletcrit (PCT); lymphocyte count (LYM), 2.6 to 13.5×10^3/μL; monocyte count (MONO), 0.0 to 0.5×10^3/μL; granulocyte count (GRAN), 0.4 to 3.2×10^3/μL; LYM%, 63.7 to 90.1%; MONO%, 1.5 to 4.5% and GRAN%, 7.3 to 30.1% were performed by auto hematology analyzer (BC-2800Vet, Mindray, Shenzhen, China).

Biochemical analysis

The second part of blood was collected into plain vials and allowed to coagulate for 1 h at room temperature followed by centrifugation at 3000×*g* for 15 min at 37 °C (R8C Remi, Laboratory centrifuge, Remi Industries Pvt. Ltd, India). The serum was separated, transferred into small test tubes for biochemical analysis. Liver function enzymes such as alanine transaminase (ALT; EC 2.6.1.2), aspartate transaminase (AST; EC 2.6.1.1), alkaline phosphatase (ALP; EC 3.1.3.1), γ-glutamyltransferase (GGT; EC 2.3.2.2) and L-lactate dehydrogenase (LDH; EC 1.1.1.27) were determined. Total serum protein (TSP), albumin (ALB), total bilirubin (TBIL) and glucose (GLU) were also assessed. Creatine kinase (CK, EC 2.7.3.2), creatinine (CRE), urea nitrogen (BUN), uric acid (UA) concentrations were determined to evaluate kidney function. The concentrations of serum triglyceride (TG) and total cholesterol (TC) were also determined to give an indication of the influence of any adverse effect of

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NCEF on the lipid profile. Serum electrolyte parameters such as calcium (Ca^{2+}), sodium (Na^{+}); potassium (K^{+}) and chlorine (Cl^{-}) were determined. All the above parameters were evaluated by using the commercial kits.

Hepatic oxidative stress analysis

Fresh liver samples of all groups were parted into two. Each portion was weighted and homogenized separately using a Potter-Elvehjem tissue homogenizer (Universal motor, RQ 127A, Remi Motors Ltd, India). One portion (10% w/v) was homogenized in 50 mM, pH 7.4 (ION 2700, pH meter 2113190, Eutech Instruments, Singapore) phosphate buffer saline (PBS). The tissue suspensions were centrifuged at 6 000×*g* for 15 min at 3 °C (LAG 412, Remi cooling centrifuge, Remi Industries Pvt. Ltd, India) to remove the cell debris, unbroken cells, nuclei and erythrocytes (Gonzalez-Flecha *et al.*, 1993). The pellet was discarded and the supernatant was used to assess the following oxidative stress markers. Determination of total hepatic protein (THP; mg/g wet tissue) was done by the modified Lowry method using bovine serum albumin (BSA) as standard (Hartree, 1972). Catalase determination (CAT; EC 1.11.1.6; U/mg protein) was performeded by spectrophotometric method (Sinha, 1972). One unit of enzyme activity is defined as the amount of catalase which catalyzed the oxidation of 1 µmol H_2O_2 per min per mg protein under assay conditions. Determination of reduced glutathione (GSH; µmol/g wet tissue) was performed by the reduction of 5, 5'dithio-bis (2-nitrobenzoic acid) (DTNB) method (Tietze, 1969). Superoxide dismutase (SOD; EC 1.15.1.1; U/mg protein) was determined by the inhibition of reduced nicotinamide adenine dinucleotide (NADH)-dependent-nitroblue tetrazolium (NBT) reduction method (Kakkar *et al.*, 1984) using a spectrophotometer at 560 nm (G9821A, Cary 100 UV-Vis Spectrophotometer, Agilent Technologies, United States). The second portion (10% w/v) was homogenized with ice-cold 150 mM KCl-Tris-HCl buffer, (pH 7.2) for the determination of malondialdehyde (MDA; nmol/g wet tissue) during lipid peroxidation using the thiobarbituric acid method (Ohkawa *et al.*, 1979) and glutathione peroxidase (GPx; EC 1.11.1.9; U/ mg protein) was determined by the method of Pinto & Bartley (1969) in which GPx was found as a result of reaction between glutathione and H_2O_2.

Table 1. Qualitative phytochemical analysis of *Neurocalyx calycinus* leaves ethanolic fraction.

Phytoconstituents		NCEF
Carbohydrates	Molish's test	+ + +
	Fehling's test	+ +
	Barfoed's test	+ +
	Benedict's test	+
Proteins and amino acids	Millon's test	+
	Biuret test	+
	Ninhydrin test	+
Alkaloids	Mayer's reagent	+
	Wagner's reagent	+ + +
	Hager's reagent	+ + +
	Dragendorff's reagent	+
Glycosides	Borntrager's test	–
	Legal's test	–
	Keller – Kiliani test	+ + +
	Kedde test	+ +
Phenolic compounds	Ferric chloride test	+ + +
	Gelatin test	+
	Lead acetate test	+ +
	Alkaline reagent test	+ + +
	Shinoda's test	+
Phytosterols	Libermann Burchard	–
	Salkowski reaction	+
Fixed oils and fats	Spot test	–
	Saponification test	–
Saponins	Foam test	–
Gum and mucilage	Alcohol 95% test	–
Volatile oils	Steam distillation	–
Iridoids	Trim- Hill reagent test	+ + +
Anthraquinones	Chloroform - 10% ammonia test	–

+ = slightly present, + + = moderately present, + + + = highly present, – = absent. All the tests were carried out three times. Observations were based on the color intensity and precipitation with appropriate reagents.

Urine analysis

At the 91st and 119th day, 24 h prior to euthanasia, the animals were housed separately in metabolic cages for urine collection. The samples were observed for color, transparency, odor and turbidity. The volume of collected urine from all animals was recorded, centrifuged at 3 000×*g* for 10 min at 4°C; supernatant was collected for the estimation of glucose (GLU); bilirubin (BIL); ketone (KET); specific gravity (SG); blood (BLO); pH; protein (PRO); urobilinogen (URO); nitrite (NIT); leukocytes (LEU) by test strips (DIRUI A10) and the sediments were analyzed microscopically for blood cells, casts, crystals, microorganisms and squamous cells.

Histopathological examination

Organs (liver, kidney, lung, spleen, heart, brain, ovary and testis) from different groups were fixed in neutral buffered 10% v/v formalin solution for histopathological assessment. After fixation, the organs were dehydrated in graded alcohol (TP1020, semi-enclosed benchtop tissue processor, Leica Biosystems, Germany), embedded in paraffin (EG1130 and EG1150, cold plate for cooling embedding molds and paraffin blocks and modular tissue embedding center, Leica Biosystems, Germany), sectioned into 4 µm thick (RM2255, fully automated rotary microtome, Leica Biosystems, Germany) and stained with Weigert's Hematoxylin and Eosin (Suvarna *et al.*, 2012). Visualization and microphotographs were captured under a light microscope (Axiostar plus 1169-149, Carl Zeiss, Germany) at magnification 10×.

Statistical analysis

All the data were expressed as mean ± standard error of the mean. The comparisons between normal control and different dose levels of drug samples treated groups were

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Table 2. Effect of *Neurocalyx calycinus* leaves ethanolic fraction on body weight and behavioral symptoms in acute oral toxicity study in Swiss albino mice.

	Female			Male			
	1st day	7th day	14th day	1st day	7th day	14th day	
Control	21.14±0.11	21.71±0.17	22.47±0.24	24.51±0.21	25.26±0.36	26.10±0.34	None
50 mg/kg	20.88±0.29ns	21.42±0.24ns	22.14±0.23ns	25.01±0.34ns	25.76±0.41ns	26.60±0.23ns	None
100 mg/kg	22.17±0.19ns	22.76±0.27ns	23.51±0.23ns	26.56±0.39ns	27.29±0.42ns	28.12±0.27ns	None
200 mg/kg	20.84±0.25ns	21.51±0.18ns	22.35±0.23ns	25.40±0.24ns	26.08±0.22ns	26.91±0.28ns	None
400 mg/kg	21.92±0.31ns	22.57±0.24ns	23.45±0.29ns	25.42±0.23ns	26.07±0.29ns	26.95±0.32ns	None
800 mg/kg	22.94±0.27ns	23.61±0.32ns	24.42±0.29ns	26.56±0.32ns	27.29±0.37ns	28.12±0.35ns	None
1600 mg/kg	21.42±0.39ns	22.03±0.27ns	22.81±0.33ns	23.90±0.13ns	24.56±0.19ns	25.36±0.21ns	None
3200 mg/kg	22.96±0.19ns	23.64±0.24ns	24.42±0.25ns	25.40±0.35ns	26.08±0.31ns	26.91±0.27ns	None
6400 mg/kg	20.17±0.11ns	20.72±0.15ns	21.43±0.20ns	23.90±0.15ns	24.56±0.24ns	25.37±0.17ns	None

Each value in the table is expressed as Mean ± SEM, n=3 ns = no significant differences (p<0.05) were observed using two-way ANOVA, followed by Dunnett's multiple comparison test.

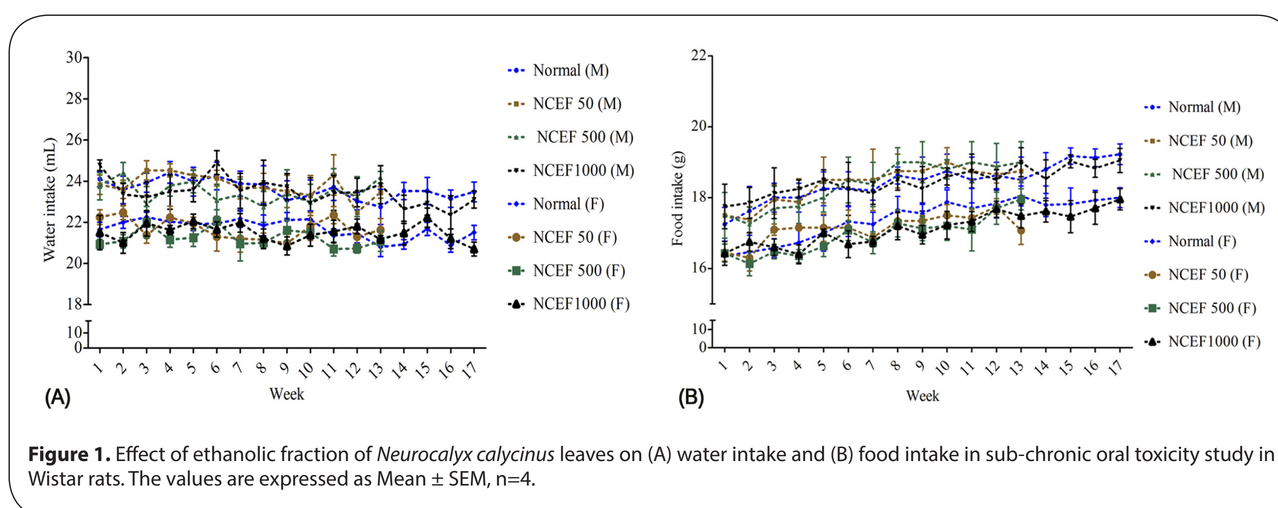

Figure 1. Effect of ethanolic fraction of *Neurocalyx calycinus* leaves on (A) water intake and (B) food intake in sub-chronic oral toxicity study in Wistar rats. The values are expressed as Mean ± SEM, n=4.

performed by two-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPAD Prism 7.01 program (Trial-version) for Windows 10 (GraphPAD, San Diego, California, USA). The p<0.05 was considered statistically significant.

Results

Qualitative phytochemical screening

The qualitative study for the phytochemical analysis of the leaf extract of *N. calycinus* (NCEF) showed the presence of carbohydrates, proteins, alkaloids, steroids, phenolic compounds and glycosides, while saponins, anthraquinones, gums, mucilages and volatile oils were not found (Table 1).

Acute oral toxicity study

The control and NCEF treated groups did not show any behavioral changes and mortality during the 14-day post-treatment period after single oral administration. The body weight of the NCEF treated animal group of both sexes exhibited statistically no significant (p<0.05) differences when compared with the control group (Table 2). Hence the median lethal dose (LD_{50}) of NCEF was greater than 6400 mg/kg b.w.

Sub-chronic oral toxicity

Behavioral observation and mortality

The control and NCEF treated animal groups showed neither mortality and nor clinical signs of toxicity in their skin, fur, eyes, mucous membranes, gait, secretions, excretions, autonomic activity, posture handling, clonic or tonic movements, stereotypes and bizarre behavior during the 90 days of repeated oral administration (dosage: 50, 500, 1000 mg/kg/b.w.) followed by 28 days of recovery period.

Food and water consumption

No changes were noted in the consumption of food and water for the animal groups treated with NCEF orally for 90 consecutive days. Hence the water intake and food consumption (Figure 1) of NCEF treated animal groups

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of both sexes showed statistically no significant (p<0.05) differences when compared with the control group.

Body weight and relative organ weight

During the 90 days of oral administration of NCEF, both male and female rats in either group did not show statistically significant differences when compared with the control group (Figure 2). The NCEF treated animal groups showed no significant variations (p<0.05) in relative organ weights as compared to control animals in both sexes (Table 3).

Hematological analysis

There were no significant (p<0.05) variations in hematological parameters in the groups treated with NCEF compared to control animals of each sex (Table 4). However, the platelet count of NCEF treated animals at doses 50, 500, 1000 mg/kg/b.w showed in both sexes statistically significant (p<0.0001) increases in dose-dependent manner when compared with the control animals during the 90 days of repeated oral administration followed by 28 days of recovery period.

Biochemical analysis

Analysis of serum biochemical parameters showed no significant (p<0.05) variations among the groups treated with NCEF and the control of the both sexes (Table 5). However, the LDH and CK levels were found significantly decreased, gradually reaching normal level during the recovery period in both sexes as compared with the normal control group.

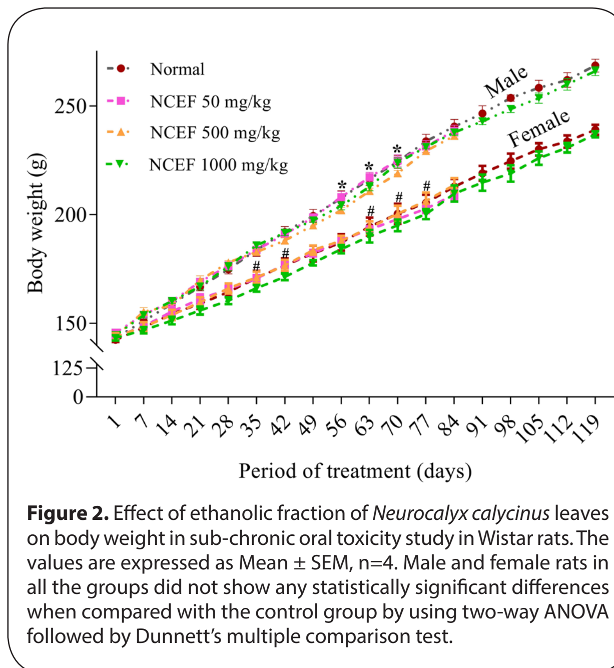

Figure 2. Effect of ethanolic fraction of *Neurocalyx calycinus* leaves on body weight in sub-chronic oral toxicity study in Wistar rats. The values are expressed as Mean ± SEM, n=4. Male and female rats in all the groups did not show any statistically significant differences when compared with the control group by using two-way ANOVA followed by Dunnett's multiple comparison test.

Table 3. Effect of *Neurocalyx calycinus* leaves ethanolic fraction on relative organ weights in sub-chronic oral toxicity study in Wistar rats.

	Treatment group				Satellite group	
#Organs	Control	NCEF 50 mg/kg	NCEF 500 mg/kg	NCEF 1000 mg/kg	Control	NCEF 1000 mg/kg
Female						
Liver	3.14 ± 0.13	3.05 ± 0.08^{ns}	2.96 ± 0.12*	2.93 ± 0.15**	3.02 ± 0.09	3.03 ± 0.14^{ns}
Kidney (L)	0.33 ± 0.01	0.32 ± 0.04^{ns}	0.32 ± 0.02^{ns}	0.34 ± 0.02^{ns}	0.32 ± 0.02	0.32 ± 0.02^{ns}
Kidney (R)	0.34 ± 0.02	0.29 ± 0.02^{ns}	0.32 ± 0.03^{ns}	0.35 ± 0.02^{ns}	0.31 ± 0.01	0.34 ± 0.01^{ns}
Lungs	0.55 ± 0.01	0.59 ± 0.02^{ns}	0.63 ± 0.06^{ns}	0.55 ± 0.04^{ns}	0.55 ± 0.02	0.54 ± 0.01^{ns}
Spleen	0.29 ± 0.04	0.32 ± 0.05^{ns}	0.37 ± 0.01^{ns}	0.31 ± 0.01^{ns}	0.35 ± 0.04	0.32 ± 0.04^{ns}
Heart	0.36 ± 0.01	0.34 ± 0.01^{ns}	0.36 ± 0.02^{ns}	0.34 ± 0.02^{ns}	0.33 ± 0.02	0.33 ± 0.01^{ns}
Brain	0.49 ± 0.01	0.55 ± 0.03^{ns}	0.53 ± 0.03^{ns}	0.52 ± 0.03^{ns}	0.4 ± 0.01	0.51 ± 0.03^{ns}
Ovary (L)	0.02 ± 0.01	0.02 ± 0.01^{ns}	0.02 ± 0.01^{ns}	0.02 ± 0.01^{ns}	0.02 ± 0.01	0.02 ± 0.01^{ns}
Ovary (R)	0.02 ± 0.01	0.02 ± 0.01^{ns}	0.02 ± 0.01^{ns}	0.02 ± 0.01^{ns}	0.02 ± 0.01	0.02 ± 0.01^{ns}
Male						
Liver	2.86 ± 0.05	2.91 ± 0.16^{ns}	2.89 ± 0.11^{ns}	2.67 ± 0.08**	2.82 ± 0.03	2.84 ± 0.14^{ns}
Kidney (L)	0.31 ± 0.02	0.30 ± 0.01^{ns}	0.32 ± 0.01^{ns}	0.32 ± 0.02^{ns}	0.30 ± 0.02	0.30 ± 0.01^{ns}
Kidney (R)	0.31 ± 0.02	0.32 ± 0.01^{ns}	0.33 ± 0.01^{ns}	0.35 ± 0.02^{ns}	0.32 ± 0.01	0.31 ± 0.02^{ns}
Lungs	0.50 ± 0.01	0.51 ± 0.02^{ns}	0.52 ± 0.02^{ns}	0.48 ± 0.01^{ns}	0.54 ± 0.05	0.47 ± 0.01^{ns}
Spleen	0.28 ± 0.02	0.29 ± 0.03^{ns}	0.23 ± 0.03^{ns}	0.29 ± 0.04^{ns}	0.27 ± 0.04	0.26 ± 0.03^{ns}
Heart	0.35 ± 0.02	0.33 ± 0.02^{ns}	0.34 ± 0.01^{ns}	0.36 ± 0.01^{ns}	0.33 ± 0.01	0.34 ± 0.01^{ns}
Brain	0.49 ± 0.03	0.49 ± 0.04^{ns}	0.46 ± 0.01^{ns}	0.46 ± 0.01^{ns}	0.44 ± 0.03	0.42 ± 0.02^{ns}
Testis (L)	0.35 ± 0.01	0.34 ± 0.01^{ns}	0.35 ± 0.02^{ns}	0.36 ± 0.01^{ns}	0.33 ± 0.01	0.34 ± 0.01^{ns}
Testis (R)	0.35 ± 0.01	0.35 ± 0.01^{ns}	0.34 ± 0.01^{ns}	0.35 ± 0.01^{ns}	0.32 ± 0.01	0.34 ± 0.01^{ns}

The values are expressed as Mean ± SEM, n=4. #g/100 g body weight. ns = non-significant, $^{*}p$<0.05, $^{**}p$<0.01 significantly different from the control group by using two-way ANOVA followed by Dunnett's multiple comparison test. L, left; R, right.

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Table 4. Effect of *Neurocalyx calycinus* ethanolic fraction of leaves on hematological parameters in sub-chronic oral toxicity study in Wistar rats.

	Treatment group				Satellite group	
Parameters	Control	NCEF 50 mg/kg	NCEF 500 mg/kg	NCEF 1000 mg/kg	Control	NCEF 1000 mg/kg
Female						
WBC (×10³/µL)	11.88±0.96	10.98±0.50ns	11.23±0.73ns	11.45±1.08ns	10.58±0.85	10.48±0.77ns
RBC (×10⁶/µL)	7.60±0.42	6.71±0.56ns	5.96±0.78ns	6.71±0.89ns	6.16±0.53	7.10±0.50ns
HGB (g/dL)	14.11±0.23	13.78±0.35ns	12.35±0.40ns	13.88±0.34ns	13.76±0.36	13.20±0.43ns
HCT (%)	40.10±2.00	36.48±3.15ns	31.52±4.11ns	35.77±4.90ns	31.96±2.68	37.16±2.89ns
MCV (fL)	52.82±0.31	54.35±0.39ns	52.91±0.32ns	53.22±0.24ns	51.91±0.25	52.25±0.43ns
MCH (pg)	18.74±1.13	20.86±1.28ns	21.71±2.51ns	21.97±3.22ns	22.71±1.49	19.01±2.02ns
MCHC (g/dL)	35.92±1.69	42.46±5.15ns	37.04±6.78ns	47.62±6.28ns	38.99±1.98	39.37±4.55ns
RDW (%)	13.55±1.05	13.15±1.64ns	15.24±1.42ns	14.28±1.23ns	15.60±0.93	13.80±0.96ns
PLT (×10³/µL)	1088.00±24.62	1125.50±32.95ns	1194.25±37.12****	1227.00±34.07****	1069.75±42.29	1209.75±39.22††††
MPV (fL)	5.05±0.18	4.78±0.13ns	4.49±0.14ns	4.20±0.04ns	5.55±0.12	4.65±0.12ns
PDW	16.45±0.80	17.05±0.50ns	15.90±0.65ns	16.75±0.35ns	16.73±0.27	16.25±0.36ns
PCT (%)	0.55±0.01	0.64±0.01ns	0.68±0.02ns	0.65±0.02ns	0.59±0.02	0.68±0.01ns
LYM (×10³/µL)	7.63±0.85	7.10±0.58ns	6.88±0.60ns	7.68±0.67ns	6.83±0.63	6.63±0.73ns
MONO (×10³/µL)	0.45±0.06	0.40±0.04ns	0.35±0.03ns	0.28±0.05ns	0.30±0.04	0.30±0.04ns
GRAN (×10³/µL)	3.80±0.24	3.48±0.36ns	4.00±0.38ns	3.50±0.53ns	3.45±0.29	3.55±0.36ns
LYM%	63.78±2.48	64.58±3.82ns	61.13±3.25ns	67.23±2.31ns	64.37±1.22	62.93±3.91ns
MONO%	3.88±0.61	3.65±0.38ns	3.11±0.14ns	2.48±0.49ns	2.99±0.64	2.89±0.41ns
GRAN%	32.34±2.03	31.77±3.47ns	35.75±3.20ns	30.29±2.70ns	32.64±1.12	34.17±3.51ns
Male						
WBC (×10³/µL)	12.20 ± 0.79	12.18 ± 0.95ns	11.48±0.40ns	10.78±0.98ns	10.93±0.99	11.05±0.37ns
RBC (×10⁶/µL)	7.85 ± 0.21	7.88 ± 0.49ns	7.21±0.47ns	6.96±0.56ns	7.16±0.66	7.10±0.50ns
HGB (g/dL)	13.86 ± 0.38	13.53 ± 0.23ns	12.60±0.23ns	13.63±0.19ns	13.26±0.26	12.95±0.56ns
HCT (%)	44.38 ± 0.76	46.08 ± 3.34ns	41.22±2.74ns	39.87±2.71ns	40.19±3.67	41.14±2.72ns
MCV (fL)	56.57 ± 0.52	58.35 ± 0.59ns	57.16±0.76ns	57.47±0.83ns	56.16±0.43	58.00±0.45ns
MCH (pg)	17.68 ± 0.58	17.39 ± 1.22ns	17.76±1.42ns	19.96±1.56ns	19.04±1.96	18.70±2.20ns
MCHC (g/dL)	31.24 ± 0.90	29.87 ± 2.36ns	31.09±2.59ns	34.64±2.28ns	33.92±3.51	32.20±3.67ns
RDW (%)	12.55 ± 0.58	11.65 ± 0.42ns	12.99±0.55ns	13.28±1.10ns	13.10±0.86	13.05±0.67ns
PLT (×10³/µL)	1088.50 ± 24.03	1110.50 ± 23.26ns	1206.25±28.14****	1244.00±24.78****	1069.50±43.49	1214.25±18.20††††
MPV (fL)	4.83 ± 0.18	4.65 ± 0.15ns	4.35±0.12ns	4.10±0.04ns	4.95±0.18	4.45±0.13ns
PDW	16.20 ± 0.60	16.80 ± 0.23ns	15.65±0.70ns	16.00±0.75ns	16.73±0.24	16.55±0.55ns
PCT (%)	0.52 ± 0.03	0.61 ± 0.01ns	0.65±0.01ns	0.62±0.01ns	0.53±0.04	0.63±0.01ns
LYM (×10³/µL)	7.83 ± 0.76	8.10 ± 0.58ns	7.78±0.47ns	7.18±0.79ns	7.08±0.65	7.38±0.48ns
MONO (×10³/µL)	0.40 ± 0.07	0.38 ± 0.06ns	0.33±0.05ns	0.35±0.06ns	0.35±0.03	0.28±0.05ns
GRAN (×10³/µL)	3.98 ± 0.14	3.70 ± 0.36ns	3.38±0.29ns	3.25±0.36ns	3.50±0.33	3.40±0.47ns
LYM%	63.72 ± 2.27	66.64 ± 1.14ns	67.69±2.72ns	66.36±2.12ns	64.74±0.79	66.86±4.32ns
MONO%	3.35 ± 0.70	3.03 ± 0.37ns	2.84±0.40ns	3.41±0.86ns	3.24±0.22	2.48±0.40ns
GRAN%	32.93 ± 0.10	30.33 ± 1.35ns	29.47±2.54ns	30.23±2.55ns	32.02±0.62	30.66±3.92ns

The values are expressed as Mean ± SEM, n=4. ns = no significant, ****p<0.0001 significantly different from the control group, ††††p<0.0001 significantly different from the satellite control group by using two-way ANOVA followed by Dunnett's multiple comparison test. WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; PLT, platelet count; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit; LYM, lymphocyte count; MONO, monocyte count; GRAN, granulocyte count. µL, microliter; dL, deciliter; fL, femtoliter; pg, picogram.

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Table 5. Effect of *Neurocalyx calycinus* leaves ethanolic fraction on serum biochemical parameters in sub-chronic oral toxicity study in Wistar rats.

	Treatment group				Satellite group	
Parameters	Control	NCEF 50 mg/kg	NCEF 500 mg/kg	NCEF 1000 mg/kg	Control	NCEF 1000 mg/kg
Female						
ALT (U/L)	35.25±1.65	33.25±2.21ns	30.75±1.75ns	32.50±1.32ns	32.25±1.65	31.00±2.58ns
AST (U/L)	64.25±2.32	65.25±1.89ns	61.00±2.65ns	65.00±2.38ns	65.50±2.75	67.25±2.78ns
ALP (U/L)	101.50±3.10	105.50±3.30ns	103.50±2.40ns	104.75±2.81ns	106.25±4.09	102.00±2.38ns
GGT (U/L)	8.25±1.31	6.25±1.11ns	8.00±1.29ns	7.75±1.11ns	9.00±1.47	7.00±1.08ns
LDH (U/L)	533.75±21.54	504.75±12.07ns	523.00±41.94ns	492.75±16.13**	549.25±30.40	522.25±39.64ns
TSP (g/L)	60.25±1.76	61.00±1.58ns	58.50±1.73ns	62.75±1.99ns	59.75±1.99	61.75±1.70ns
ALB (g/L)	49.75±1.49	48.25±1.25ns	47.25±1.93ns	46.25±1.55ns	45.25±1.25	47.75±1.84ns
TBIL (µmol/L)	4.90±0.14	5.03±0.09ns	4.80±0.13ns	4.85±0.16ns	5.00±0.16	4.95±0.14ns
CK (U/L)	360.50±17.49	348.75±26.22ns	380.75±32.02ns	320.75±24.99**	387.00±21.30	344.05±17.08ns
CRE (µmol/L)	44.90±2.27	46.74±3.53ns	45.93±2.42ns	47.08±3.58ns	42.75±3.47	44.25±3.33ns
BUN (mmol/L)	14.85±0.59	14.28±0.84ns	13.68±0.39ns	15.45±0.72ns	13.53±0.49	14.95±0.43ns
UA (µmol/L)	38.50±4.03	34.50±2.47ns	36.00±5.40ns	42.25±3.35ns	36.50±4.21	39.00±4.55ns
GLU (mmol/L)	6.43±0.13	6.28±0.11ns	6.30±0.18ns	6.23±0.13ns	6.18±0.19	6.06±0.12ns
TC (mmol/L)	2.06±0.23	2.19±0.19ns	2.34±0.28ns	1.96±0.12ns	2.16±0.12	2.05±0.12ns
TG (mmol/L)	0.95±0.08	0.96±0.10ns	0.84±0.06ns	0.94±0.08ns	0.91±0.06	0.95±0.09ns
Ca (mmol/L)	0.89±0.11	0.81±0.07ns	0.88±0.05ns	0.83±0.07ns	0.86±0.05	0.86±0.11ns
Na (mmol/L)	133.00±3.76	138.00±3.34ns	133.25±5.62ns	133.75±3.17ns	137.25±4.52	135.75±4.42ns
K (mmol/L)	3.36±0.10	3.56±0.21ns	3.72±0.28ns	3.61±0.25ns	3.66±0.19	3.77±0.15ns
Cl (mmol/L)	97.30±4.27	96.75±4.33ns	99.00±3.03ns	98.75±3.57ns	97.50±3.30	102.50±2.02ns
Male						
ALT (U/L)	39.75±1.11	34.75±0.85ns	33.50±1.85ns	36.75±2.02ns	37.50±1.71	35.50±2.72ns
AST (U/L)	61.50±1.71	63.25±1.11ns	62.50±2.02ns	59.75±3.01ns	62.25±1.65	63.25±3.15ns
ALP (U/L)	96.50±2.50	100.50±2.63ns	95.75±4.37ns	103.00±3.76ns	99.75±2.32	102.75±2.95ns
GGT (U/L)	11.50±1.44	10.25±1.31ns	10.75±1.25ns	13.50±1.71ns	11.75±1.38	9.75±1.55ns
LDH (U/L)	708.75±31.87	729.50±24.84ns	730.50±41.56ns	678.25±49.44*	723.25±31.30	724.50±27.32ns
TSP (g/L)	55.25±1.66	54.80±1.87ns	56.00±1.98ns	55.50±1.46ns	55.12±1.21	54.50±1.54ns
ALB (g/L)	42.00±1.29	41.00±1.22ns	42.50±1.85ns	40.25±1.75ns	41.75±1.38	42.25±1.55ns
TBIL (µmol/L)	4.60±0.13	4.43±0.23ns	4.38±0.17ns	4.40±0.18ns	4.62±0.13	4.48±0.20ns
CK (U/L)	437.25±21.58	429.50±12.69ns	435.25±15.70ns	399.75±14.10*	436.50±16.29	430.25±24.50ns
CRE (µmol/L)	39.55±2.27	42.05±2.80ns	43.13±3.45ns	38.43±3.17ns	40.30±2.49	37.55±2.68ns
BUN (mmol/L)	14.03±0.34	13.90±0.42ns	14.38±0.40ns	14.53±0.39ns	13.63±0.37	14.73±0.89ns
UA (µmol/L)	32.65±2.83	35.00±3.11ns	33.75±3.84ns	36.50±3.62ns	36.50±2.99	38.50±3.88ns
GLU (mmol/L)	6.40±0.12	6.53±0.11ns	6.60±0.13ns	6.45±0.10ns	6.56±0.13	6.43±0.15ns
TC (mmol/L)	1.86±0.20	1.63±0.18ns	1.71±0.25ns	1.83±0.17ns	1.77±0.19	1.80±0.16ns
TG (mmol/L)	1.35±0.18	1.28±0.11ns	1.24±0.10ns	1.35±0.12ns	1.33±0.08	1.39±0.10ns
Ca (mmol/L)	0.77±0.08	0.71±0.06ns	0.78±0.09ns	0.77±0.06ns	0.78±0.04	0.72±0.10ns
Na (mmol/L)	131.00±2.80	132.75±3.77ns	135.25±4.96ns	139.00±3.24ns	135.50±3.80	128.25±4.35ns
K (mmol/L)	3.37±0.11	3.59±0.18ns	3.69±0.27ns	3.50±0.23ns	3.90±0.19	3.44±0.23ns
Cl (mmol/L)	95.35±3.65	89.25±4.33ns	91.00±2.12ns	100.00±4.42ns	97.00±3.42	96.00±3.92ns

The values are expressed as Mean ± SEM, n=4. ns = no significant differences ($p<0.05$) were observed using two-way ANOVA, followed by Dunnett's multiple comparison test. *$p<0.05$, **$p<0.01$ significantly different from the control group by using two-way ANOVA followed by Dunnett's multiple comparison test. ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, γ-glutamyltransferase; LDH, lactate dehydrogenase; TSP, total serum protein; ALB, albumin; TBIL, total bilirubin; GLU, glucose; CK, creatine kinase; CRE, creatinine; BUN, urea nitrogen; UA, uric acid; TG, triglyceride; TC, total cholesterol; Ca, calcium; Na, sodium; K, potassium; Cl, chlorine. U/L, units per litre; g/L, gram per litre; µmol/L, micromoles/litre; mmol/L, millimole per litre.

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Hepatic oxidative stress analysis

The hepatic oxidative stress marker index (CAT, GSH, SOD, MDA and GPx) of orally administered NCEF treated groups of female and male Wistar rats is shown in Figure 3. A dose-dependent non-significant increase (p<0.05) in total protein content (Figure 3A) and a dose-dependent decrease in CAT activity were observed in both female and male rats (Figure 3B). However, a significant reduction (p<0.01) was observed in female rats at NCEF 1000 mg/kg when compared to the control group, which was normalized after the recovery period. A dose-dependent increase in GSH activity was found in both female and male rat (Figure 3C). Further, a significant increase was observed in female rats at NCEF 500 mg/kg (p<0.05), 1000 mg/kg (p<0.01) and male rats at NCEF 1000 mg/kg (p<0.05) as compared to the control group, which was seen normalized during the recovery period. A dose-dependent increase in SOD activity was observed in both female and male rats (Figure 3D). However, a significant increase (p<0.05) was observed in female rats at NCEF 1000 mg/kg, as compared to the control group, which became regularized after the recovery period. A dose-dependent non-significant increase (p<0.05) in MDA level was observed in both female and male rats (Figure 3E). A dose-dependent increase in GPx activity was observed in both female and male rats (Figure 3F). A significant increase in GPx was observed in female rats at NCEF 500 mg/kg (p<0.05), 1000 mg/kg (p<0.01) and in male rats at NCEF 1000 mg/kg (p<0.01) as compared to the control group, which was normalized after the recovery period.

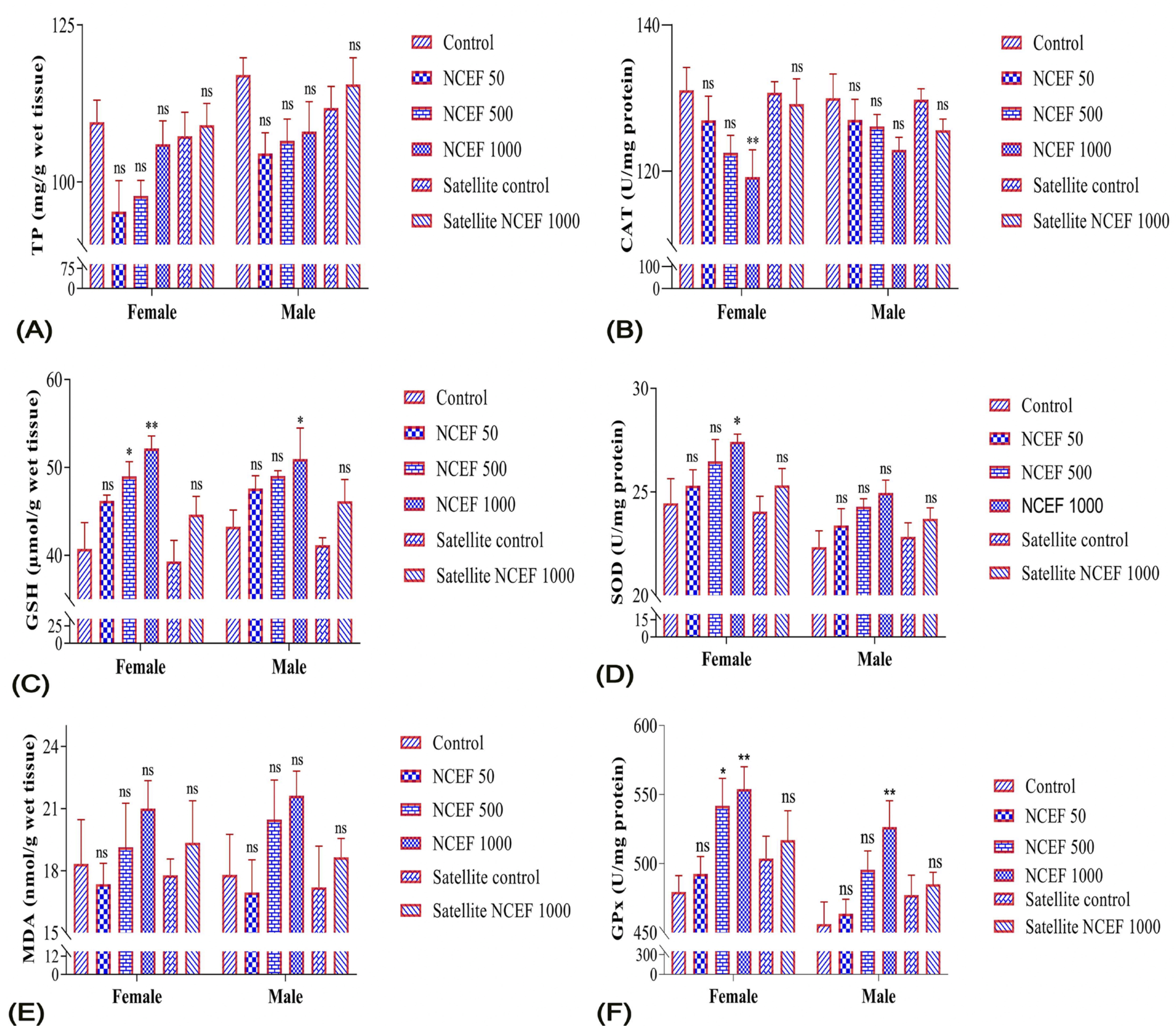

Figure 3. Effect of ethanolic fraction of *Neurocalyx calycinus* leaves on hepatic biochemical markers for oxidative stress in sub-chronic oral toxicity study of both female and male Wistar rats. (A) TP (total protein) expressed as mg/g (milligram/gram) wet tissue; (B) CAT (catalase activity expressed as U/mg (units/milligram) protein; (C) GSH (reduced glutathione) activity expressed as µmol/g (micromole per gram) wet tissue; (D) SOD (superoxide dismutase) activity expressed as U/mg protein; (E) MDA (malondialdehyde) activity expressed as nmol/g (nanomole/gram) wet tissue; (F) GPx (glutathione peroxidase) activity expressed as U/ mg protein). The results are expressed as Mean ± SEM, n=4. ns, no significant differences (p<0.05); *p<0.05, **p<0.01 significantly different from the control group by using two-way ANOVA followed by Dunnett's multiple comparison test.

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Urine analysis

There were no significant changes in the urinary parameters observed in the group treated with NCEF compared to controls of both sexes (Table 6). Urine samples showed yellow color and a characteristic odor. Blood cells, casts, crystals, microorganisms and epithelial cells were not seen in the microscopic examination of urine samples collected from the NCEF treated rats.

Histopathological examination

There were no irregularities in the structural integrity of organs observed in the histopathological examination after 90 days of oral administration of NCEF 50, 500 and 1000 mg/kg dose groups. Microphotographs of the liver showed normal sinusoids, portal vein and hepatocytes, which are radially arranged from the central vein. Absence of fatty cells, cytoplasmic vacuolation, necrosis and minimum of Kupffer cell infiltration indicates that NCEF is safe for oral administration (Figures 4.1, a–d). Cross section of the kidney showed normal architecture. Tubular necrosis and lymphocyctic infiltration were absent in the entire NCEF treated groups (Figures 4.2, a–d). The myocardium layer of the heart showed normal cardiac muscle fibers and capillaries. Infiltration of inflammatory cells, fibrosis and ganglionic abnormalities were not detected (Figures 4.3, a–d). Lung tissues showed normal delicate alveolar septa, uniform distribution of alveoli, bronchioles lined by single ciliated epithelium. No septal breakage, alveoli expansion and bronchiolar inflammation were observed (Figures 4.4, a–d). Brain histologic examination showed normal architecture in the cerebral cortex. No necrosis and vacuolar changes were found (Figures 4.5, a–d). Spleen histology showed no differences in the splenic cords, sinusoids and central arteriole (Figures 4.6, a–d). Histology of the ovary showed normal architecture in cortex and medullary

Table 6. Effect of *Neurocalyx calycinus* ethanolic fraction of leaves on urine analysis in sub-chronic oral toxicity study in Wistar rats.

	Treatment group				Satellite group	
Parameters	**Control**	**NCEF 50 mg/kg**	**NCEF 500 mg/kg**	**NCEF 1000 mg/kg**	**Control**	**NCEF 1000 mg/kg**
Female						
*Volume (mL/24 h)	6.84±1.78	6.45±2.00^{ns}	6.01±2.14^{ns}	5.37±1.04^{ns}	6.92±1.57	6.17±2.64^{ns}
GLU	–	–	–	–	–	–
BIL	–	–	–	+	–	–
KET	–	–	+	+	–	–
SG	1.00	1.00	1.00	1.00	1.00	1.00
BLO	–	–	–	–	–	–
pH	1#	1#	1#	1#	1#	1#
PRO	++	++	++	+++	++	++
LEU	t	t	t	t	t	t
NIT	–	–	–	–	–	–
URO	–	–	–	–	–	–
*CRE (µmol/L)	124.47±9.35	105.11±14.58^{ns}	112.58±17.43^{ns}	139.84±12.45^{ns}	127.65±11.26	131.72±10.87^{ns}
Male						
*Volume (mL/24 h)	5.29±0.98	5.86±1.76^{ns}	6.25±1.07^{ns}	5.99±2.40^{ns}	5.07±1.88	5.59±2.01^{ns}
GLU	–	–	–	–	–	–
BIL	–	–	+	+	–	–
KET	–	–	–	+	–	–
SG	1.00	1.00	1.00	1.00	1.00	1.00
BLO	–	–	–	–	–	–
pH	1#	1#	1#	1#	1#	1#
PRO	+	++	++	+++	++	++
LEU	t	t	t	t	t	t
NIT	–	–	–	–	–	–
URO	–	–	–	–	–	–
*CRE (µmol/L)	104.36±8.24	111.27±12.35^{ns}	98.75±16.25^{ns}	109.77±11.21^{ns}	96.41±15.64	101.89±13.78^{ns}

*The values are expressed as Mean ± SEM, n=4. ns = no significant differences ($p<0.05$) were observed using two-way ANOVA, followed by Dunnett's multiple comparison test. #pH = between 6 and 9. µmol/L, micromoles/litre; GLU, Glucose; BIL, bilirubin; KET, ketone; SG, specific gravity; BLO, blood; PRO, protein; LEU, leukocytes; NIT, nitrite; URO, urobilinogen (URO); CRE, creatinine. + = slightly present, + + = moderately present, + + + = highly present, – = absent, t = trace.

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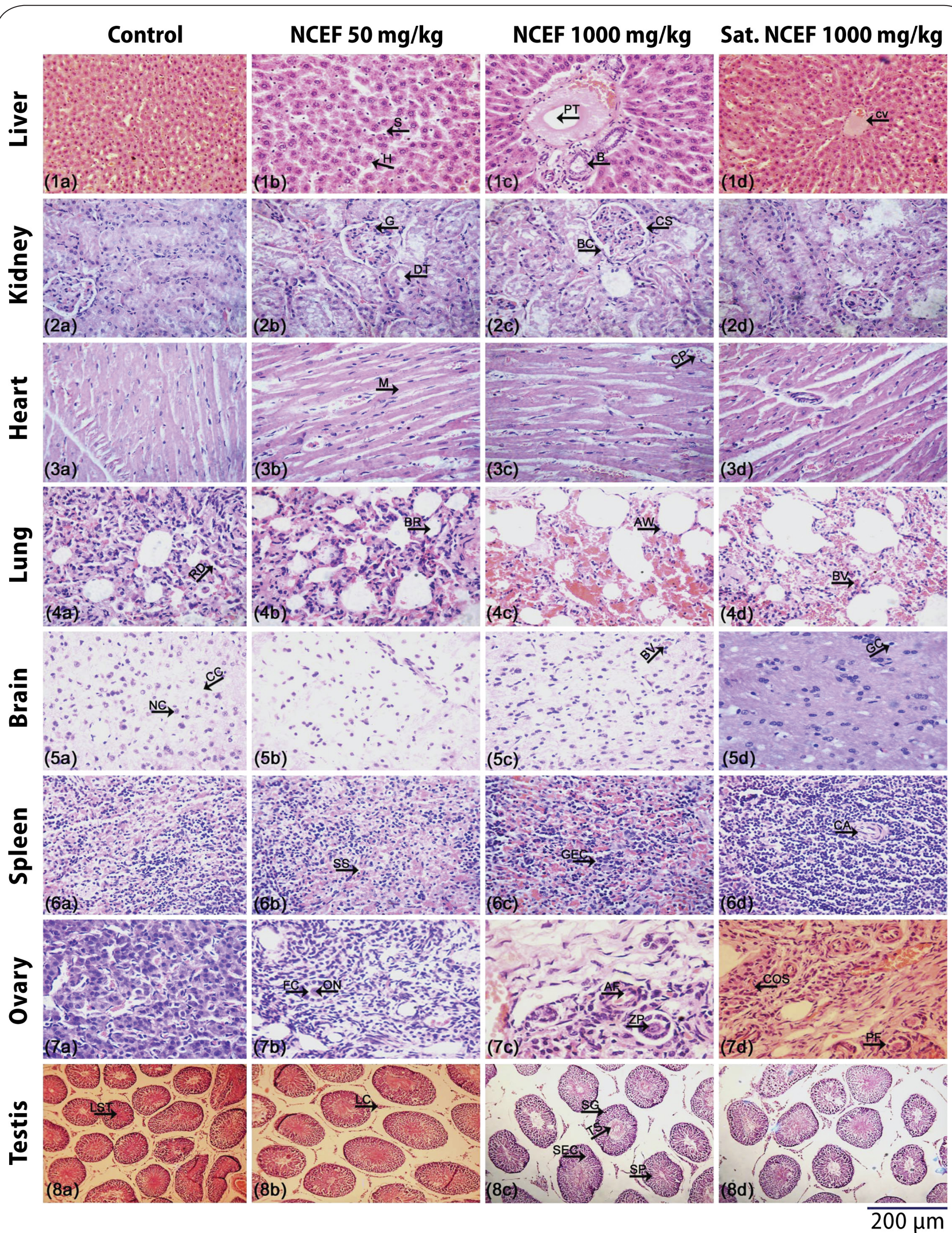

Figure 4. Microphotographs of histopathological evaluation of organs in Wistar rats treated with leaves ethanolic fraction of *Neurocalyx calycinus* in sub-chronic oral toxicity study. Histological sections of selected groups stained with hematoxylin and eosin a. Control, b. NCEF 50 mg/kg, c. NCEF 1000 mg/kg, d. Satellite 1000 mg/kg. (1a-1d) Liver, (2a-2d) Kidney (right), (3a-3d) Heart, (4a-4d) Lung, (5a-5d) Brain, (6a-6d) Spleen, (7a-7d) Ovary (right) and (8a-8d) Testis (right). S = Sinusoid; H = Hepatocyte; PT = Portal vein; B = Bile duct; CV = Central Vein; G = Glomerulus; DT = Distal tubules; BC = Bowman's capsule; CS = Capsular space; M = Myofibrils; CP = Capillaries; RD = Respiratory ducts; BR = Bronchioles; AW = Alveolar walls; BV = Blood vessel; CC = Cerebral cortex; NC = Neuronal cells; GC= Glial cell; SS = Splenic sinuses; GEC = Germinal center; CA = Central arteriole; FC = Follicular cavity; ON = Oocyte nuclei; AF = Atretic follicle; ZP = Zona pellucida; COS = Cortical stroma, PF = Primary follicle; LST = Lumen of seminiferous tubule; LC = Leydig cell; SG = Spermatogonium; TS = Tails of spermatozoa; SEC = Sertoli cell; SP = Spermatozoon.

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region. Primordial follicles, thick-walled blood vessels and rete cells were normally functioning (Figures 4.7, a–d). Normal interstitial tissues, Leydig cells, Sertoli cells, seminiferous tubules, primary spermatocytes are seen in the testis (Figures 4.8, a–d).

Discussion

The leaves of *N. calycinus* belonging to the family Rubiaceae were extensively used by the Cholanaickan tribe of Kerala for treating wounds and inflammations. The worldwide acceptance of plant-based medicines is possible only if they fulfil the same efficacy, quality and safety parameters as synthetic products (Rates, 2001). Based on these assumptions, our present study focuses on acute and 90-day sub-chronic toxicity studies for understanding the impacts of long-term consumption of *N. calycinus* leaves as medicine.

In the present study, different solvent extracts of leaves of *N. calycinus* were checked for various *in vivo* and *in vitro* experiments. The ethanolic fraction showed the most promising activity. Preliminary phytochemical screening of NCEF of leaves showed the presence of major phytocompounds like alkaloids, glycosides, iridoids and phenolic compounds like coumarins, flavonoids and tannins. The presence of these compounds depends upon the environmental conditions, the plant part and the extraction solvents used for the study. The presence of phenols is considered to be potentially toxic to the growth and development of pathogens. Although anthraquinones, iridoids and alkaloids are the secondary metabolites in Rubioideae (Martins & Nunez, 2015), anthraquinones were absent in NCEF.

Toxicity studies are the essential criteria for pharmacological evaluation and standardization of unknown substances. Acute oral toxicity study of NCEF showed no mortality even at its highest dose level (6400 mg/kg b.w.). Acute toxicity studies provide information about the therapeutic index and fix the absolute dose for pharmacological tests in rodents (Chinedu *et al*., 2013). The chemical labeling and classification of acute toxicity based on oral LD_{50} values recommended by the OECD guideline 423 (OECD, 2002) also showed LD_{50} value for ethanolic fraction of *N. calycinus* leaves to be greater than 5000 mg/kg body weight, thus coming under category 5. Body weight is one of the most sensitive indicators of the condition of an animal if it is monitored frequently and carefully during the study. In the toxicity studies no difference in body weight was found at any of the doses throughout the experiment. Body weight is measured at least once a week during toxicity studies. A treatment dose which causes more than 10% reduction in body weight is considered to be toxic (Kushwaha *et al*., 2013). Our results confirmed that the extract did not possess any acute oral toxicity in mice.

Based on the acute toxicity data, a lower (50 mg/kg bw), middle (500 mg/kg bw) and higher (1000 mg/kg bw) dose of the NCEF were selected for sub-chronic oral toxicity study in both sexes of Wistar rats. In the sub-chronic oral toxicity study, NCEF did not induce any signs of morbidity and mortality, and that not even in the group treated with the highest dose (1000 mg/kg bw) during the 90 days of treatment and 28 days of recovery period.

Water intake, food consumption, body weight and relative organ weight are interrelated and are the primary indicators of adverse effects of a toxic substance in rodents (Speakman & McQueenie, 1996; Shokryazdan *et al*., 2016). Any variation in food consumption and water intake will directly affect the normal metabolism of animals (Mukinda & Syce, 2007). Any changes in body weight can be used as a rapid assessment for side effects of a drug (Teo *et al*., 2002). In the present study, female and male rats of all the NCEF treated groups showed a regular intake of water and food similar to the control rats. Increase in body weight of NCEF treated animals was not significantly different from the control group. This general increase may be due to the quality of food and water. These results confirmed that NCEF did not affect growth retardation and appetite of rats. The mean relative organ weight of kidney, lungs, spleen, heart, brain, testis and ovary did not show any significant differences when compared to the control group. However, the liver of both female and male rats showed a significant decrease in weight at the highest dose of NCEF (1000 mg/kg b.w.). Understanding the organ to body weight ratio on studying herbal products is mandated to identify the target organs, mechanism of action and toxicokinetics (Bailey *et al*., 2004; Sellers *et al*., 2007; Adewale *et al*., 2016). Based on these findings, NCEF is safe and non-toxic to animals for long-term oral administration.

Blood cells, the most sensitive connective tissue in mammals, responsible for the transport of nutrients and foreign bodies, are the target areas of toxic substances. Any damage in these cells will directly indicate the physiological condition, inflammations and infections of the animals (Petterino & Argentino-Storino, 2006; Ezeja *et al*., 2014). No significant variations were observed in any of the blood parameters of NCEF treated groups and control groups in both sexes, except in the platelet count. A significant dose-dependent increase in platelet count of both female and male rats was observed during the 90 days of treatment , and even after the recovery period. The increases in platelet count might be an indication of the platelet augmentation property of *N. calycinus*. Further studies are required to scientifically validate the platelet augmentation effect of the plant. Increased platelet count plays a significant role in hemostasis (Anitua *et al*., 2004) and also the release of potent growth factors for repairing various wounds (Knighton *et al*., 1986).

The liver is the vital organ for drug biotransformation. Abnormalities present in the hepatic cells will affect the normal detoxification mechanism of animals. A wide variety of toxic substances is extensively biotransformed into metabolites with markedly different toxicological properties. Such biotransformation takes variable times in different tissues and different species, convolving the cellular toxicological effects (Nicholson *et al*., 2002). Serum

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biochemical studies will provide a significant inference about the nature of toxic effects on the liver (Wolf, 1999). Oral administration of NCEF did not cause any significant changes in the serum enzyme levels of ALT, AST, ALP and GGT as compared to control rats of both sexes. However, LDH level was significantly reduced in both female and male rats at the highest dose (NCEF 1000 mg/kg bw). The standard level of the LDH was regained after 28 days of the recovery period. An increase in the level of serum markers will give information about the nature and type of chronic liver damage (Sheweita *et al*., 2001; Ramaiah, 2007). Among these parameters, ALT enzymatic activity is highly sensitive (Ozer *et al*., 2008). However, NCEF oral consumption did not elevate the enzyme level in all dose groups. Other serum parameters such as TSP, ALB and TBIL showed no significant changes as compared to normal rats of both sexes, indicating that NCEF did not cause any adverse effects in animals. Hepatotoxic substances change the standard level of serum enzymes (ALT, AST, ALP, GGT and LDH), which are vital diagnostic parameters in liver disease and cell injury (Galle *et al*., 1990; Sutcu *et al*., 2006). In the present study, no variations were observed in the CRE, BUN, UA and electrolyte levels such as Na, K, Cl and Ca. The CRE levels were also found to be normal during the recovery period of 28 days. These serum biochemical markers are prominent indicators of kidney function and any fluctuations in the normal level of these parameters directly reflect the metabolism (Vasan, 2006). Our study suggests that NCEF has no adverse effect on hepatic and renal cells, indicating its non-toxicity. There were no significant changes observed in the GLU, TC and TG levels of NCEF treated rats compared to control rats. The non-significant difference of hepatic and renal serum marker levels (Hilaly *et al*., 2004) between the control and treated groups suggests that repeated daily oral consumption of NCEF did not alter the normal biologic process of animals. Furthermore, detailed molecular profiling like genomics, proteomics, and metabolomics analysis (Hellmold *et al*., 2002) is desirable to understand the effect of oral consumption of NCEF on whole-organism functional integrity overtime after drug exposure.

The liver plays a critical role in the maintenance of body homoeostasis. The imbalance between the excess formation of pro-oxidants and the antioxidant defence mechanism of a cell leads to oxidative stress, which causes tissue damage and cell death (Sanders, 2001; Mates *et al*., 2008; Weydert & Cullen, 2009). No significant difference was observed in the activity of antioxidant enzymes (CAT, GPx and SOD) together with GSH and the level of oxidation product MDA in any of the groups studied. SOD helps the endogenous antioxidant defence system to scavenge radicals and maintain cellular redox balance. GPx catalyzes the reduction of H_2O_2 and other peroxides. GSH protects cells against lipid peroxidation (Yao *et al*., 2007). In the present study, we confirmed that repeated 90-day oral consumption of NCEF did not induce any oxidative stress and liver toxicity and normal hepatic non-enzymatic and enzymatic antioxidant status was maintained in rats.

Kidney is one of the primary targets of toxic substances. Urinary parameters, especially BUN and CRE, assist in the detection of renal failure (Hoffmann *et al*., 2010). Physicochemical and microscopical urine test will give vital information about the abnormalities in renal and urinary tract diseases. But, there was no significant difference in the urinary parameters between the NCEF treated and control animals. However, in the animals of either sex treated with highest dose, the urine color changed to pale yellowish. The microscopic examination of urine sediments did not find blood cells, casts, crystals, squamous cells and microorganisms. A negative nitrite test confirmed the absence of microorganisms.

The vital organs are extremely sensitive to toxic elements, and the resulting hypertrophy of these organs provides a direct indication of toxicity which may be useful in predicting toxicity level, enzyme induction, physiologic perturbations and acute injury (Michael *et al*., 2007). Histopathological examination of the liver, kidneys, lungs, spleen, heart, brain, ovaries and testes after repeated 90-day oral consumption of NCEF did not show any anatomical disorder and detrimental changes at any dose level compared with normal rats. No signs of inflammations, lesions, color changes, texture and hypertrophy confirmed the non-toxic nature of NCEF. In our biochemical observations slight variations of normal parameters had no effect on the routine function of vital organs, which was corroborated by histological findings. These findings will help to fix dose-dependent safety margins for using NCEF as a drug and its role in targeted visceral organs (Kramer *et al*., 2007).

Conclusion

The sub-chronic toxicity effect of ethanolic fraction of leaves of *Neurocalyx calycinus* will be a stepping stone for various preclinical and clinical pharmacological studies in future. The acute toxicity study at NCEF 6400 mg/kg/b.w. did not cause any mortality and adverse effects. Repeated oral consumption of NCEF for 90 days among male and female Wistar rats did not show any deleterious changes in food and water consumption, body weight, organ weight, hematological, biochemical, oxidative stress and histopathological parameters. Therefore, based on these findings, it is confirmed for the first time that prolonged oral consumption of NCEF is safe, non-toxic and also not adversely affecting the normal physiological functions of the animal. Our present findings also facilitate the future step for the development of a potent drug candidate in nearby future from the leaves of *N. calycinus*.

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REFERENCES

Adewale OB, Onasanya A, Anadozie SO, Abu MF, Akintan IA, Ogbole CJ, Olayide II, Afolabi OB, Jaiyesimi KF, Ajiboye BO, Fadaka AO. (2016). Evaluation of acute and subacute toxicity of aqueous extract of *Crassocephalum rubens* leaves in rats. *J Ethnopharmacol* **188**: 153–158.

Aneesh TP, Hisham M, Sekhar S, Madhu M, Deepa TV. (2009). International market scenario of traditional Indian herbal drugs – India declining. *Int J green pharm* **3**: 184.

Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. (2004). Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost* 91: 4–15.

Bailey SA, Zidell RH, Perry RW. (2004). Relationships between organ weight and body/brain weight in the rat: What is the best analytical Endpoint? *Toxicol Pathol* **32**: 448–466.

Bremer B. (1979). The genus *Neurocalyx* (Rubiaceae– Argostemmateae) in Ceylon. *Bot Not* **132**: 399–407.

Bremer B. (1987). Genus *Neurocalyx*, in: Dassanayake MD, Fosberg FR. (Eds.), A revised handbook to the flora of Ceylon. Oxford and IBH publishing Co. Pvt. Ltd., New Delhi. 322–326.

Chinedu E, Arome D, Ameh FS. (2013). A new method for determining acute toxicity in animal models. *Toxicol Int* **20**: 224–226.

Ezeja MI, Anaga AO, Asuzu IU. (2014). Acute and sub-chronic toxicity profile of methanol leaf extract of *Gouania longipetala* in rats. *J Ethnopharmacol* **151**: 1155–1164.

Fabricant DS, Farnsworth NR. (2001). The value of plants used in traditional medicine for drug discovery. *Environ Health Perspect* **109**: 69–75.

Galle PR, Theilmann L, Raedsch R, Otto G, Stiehl A. (1990). Ursodeoxycholate reduces hepatotoxicity of bile salts in primary human hepatocytes. *Hepatology* **12**: 486–491.

Gonzalez-Flecha B, Evelson P, Sterin-Speziale N, Boveris A. (1993). Hydrogen peroxide metabolism and oxidative stress in cortical, medullary and papillary zones of rat kidney. *Biochim Biophys Acta Gen Subj* **1157**: 155–161.

Harborne JB. (1998). Phytochemical methods: A guide to modern techniques of plant analysis, 3rd ed. Springer-Verlag, New York.

Hartree EF. (1972). Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Anal Biochem* **48**: 422–427.

Hashmi S, Singh VK. (2003). Importance of Pharmacognosy as an aid to drug standardisation programme: a review, in: Singh VK, Govil JN, Hashmi S, Singh G. (Eds.), Recent progress in medicinal plants: Ethnomedicine and Pharmacognosy-II, volume 7. Studium press LLC., United States. 339–346.

Hellmold H, Nilsson CB, Schuppe-Koistinen I, Kenne K, Warngard L. (2002). Identification of end points relevant to detection of potentially adverse drug reactions. *Toxicol Lett* **127**: 239–243.

Hilaly JE, Israili ZH, Lyoussi B. (2004). Acute and chronic toxicological studies of Ajuga iva in experimental animals. *J Ethnopharmacol* **91**: 43–50.

Hoffmann D, Adler M, Vaidya VS, Rached E, Mulrane L, Gallagher WM, Callanan JJ, Gautier JC, Matheis K, Staedtler F, Dieterle F, Brandenburg A, Sposny A, Hewitt P, Ellinger-Ziegelbauer H, Bonventre JV, Dekant W, Mally A. (2010). Performance of novel kidney Biomarkers in preclinical toxicity studies. *Toxicol Sci* **116:** 8–22.

Kakkar P, Das B, Viswanathan PN. (1984). A modified spectrophotometric assay of superoxide dismutase. *Indian J Biochem Biophys* **21**: 130–132.

Knighton DR, Ciresi KF, Fiegel VD, Austin LL, Butler EL. (1986). Classification and treatment of chronic non-healing wounds. Successful treatment with autologous platelet-derived wound healing factors (PDWHF). *Ann Surg* **204**: 322–330.

Kramer JA, Sagartz JE, Morris DL. (2007). The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. *Nat Rev Drug Discov* **6**: 636–649.

Kushwaha SK, Dashora A, Dashora N, Patel JR, Kori ML. (2013). Acute oral toxicity studies of the standardized methanolic extract of *Phyllanthus amarus* Schum & Thonn. *J Pharm Res* **6**: 720–724.

Lorke D. (1983). A new approach to practical acute toxicity testing. *Arch. Toxicol.* **54**: 275–287.

Martins D, Nunez CV. (2015). Secondary metabolites from Rubiaceae species. *Molecules* **20**: 13422–13495.

Mates JM, Segura JA, Alonso FJ, Marquez J. (2008). Intracellular redox status and oxidative stress: Implications for cell proliferation, apoptosis, and carcinogenesis. *Arch Toxicol* **82**: 273–299.

Mathur PRG. (2013). Traditional Knowledge of the Cholanaickan and Kurumba: The hunter gatherers of Kerala. *J Traditional Folk practices* **1**: 19–30.

Menon M. (1996). The Encyclopaedia of Dravidian Tribes, second vol. The International school of Dravidian Linguistics, Thiruvananthapuram, India.

Michael B, Yano B, Sellers RS, Perry R, Morton D, Roome N, Johnson JK, Schafer K, Pitsch S. (2007). Evaluation of organ weights for rodent and non-rodent toxicity studies: A review of regulatory guidelines and a survey of current practices. *Toxicol. Pathol* **35**: 742–750.

Mukinda JT, Syce JA. (2007). Acute and chronic toxicity of the aqueous extract of artemisia afra in rodents. *J Ethnopharmacol.* **112**: 138–144.

Nicholson JK, Connelly J, Lindon JC, Holmes E. (2002). Metabonomics: A platform for studying drug toxicity and gene function. *Nat Rev Drug Discov* **1**: 153–161.

OECD (The Organisation of Economic Co-operation Development)., 1998. Test guidelines No. 408: Repeated dose 90-day oral toxicity study in Rodents, OECD Publishing, Paris.

OECD (The Organisation of Economic Co-operation Development)., 2002. Test guidelines No. 423: Acute Oral toxicity – Acute Toxic Class Method, OECD Publishing, Paris.

Ohkawa H, Ohishi N, Yagi K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* **95**: 351–358.

Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology* **245**: 194–205.

Petterino C, Argentino-Storino A. (2006). Clinical chemistry and haematology historical data in control Sprague-Dawley rats from pre-clinical toxicity studies. *Exp Toxicol Pathol* **57**: 213–219.

Pinto RE, Bartley W. (1969). The effect of age and sex on glutathione reductase and glutathione peroxidase activities and on aerobic glutathione oxidation in rat liver homogenates. *Biochem J* **112**: 109–115.

Ramaiah SK. (2007). A toxicologist guide to the diagnostic interpretation of hepatic biochemical parameters. Food and Chemical Toxicology **45**: 1551–1557.

Rates SM. (2001). Plants as source of drugs. *Toxicon* **39**: 603–613.

Sanders RA, Rauscher FM, Watkins JB. (2001). Effects of quercetin on antioxidant defense in streptozotocin-induced diabetic rats. *J Biochem Mol Toxicol* **15**: 143–149.

Saradamma L, Nair CPR, Bhat AV, Rajasekharan S. (1994). Final technical Report: All India Co-ordinate Research Project on Ethnobiology-Phase II.

Sellers RS, Morton D, Michael B, Roome N, Johnson JK, Yano BL, Perry R, Schafer K. (2007). Society of Toxicologic pathology position paper: Organ weight recommendations for toxicology studies. *Toxicol Pathol* **35**: 751–755.

Sheweita SA, El-Gabar MA, Bastawy M. (2001). Carbon tetrachloride-induced changes in the activity of phase II drug-metabolizing enzyme in the liver of male rats: Role of antioxidants. *Toxicology* **165**: 217–224.

Shokryazdan P, Jahromi MF, Liang JB, Kalavathy R, Sieo CC, Ho YW. (2016). Safety assessment of Two new Lactobacillus strains as Probiotic for human using a rat model. *PLOS ONE* **11**: e0159851.

Sinha AK. (1972). Colorimetric assay of catalase. *Anal Biochem* **47**: 389–394.

Sofowora A. (1982). Medicinal plants and traditional medicine in Africa. John Wiley and Sons, United Kingdom.

Speakman JR, McQueenie J. (1996). Limits to sustained metabolic rate: The link between food intake, Basal metabolic rate, and morphology in reproducing mice, mus musculus. *Physiol Zool.* **69**: 746–769.

Sutcu R, Altuntas I, Yildirim B, Karahan N, Demirin H, Delibas N. (2006). The effects of subchronic methidathion toxicity on rat liver: Role of antioxidant vitamins C and E. *Cell Biol Toxicol* **22**: 221–227.

Suvarna KS, Layton C, Bancroft JD. (2012). Bancroft's theory and practice of histological techniques, 7th ed. Elsevier Science Health Science div, Oxford.

Takhtajan A. (2009). Flowering plants, 2nd ed. Springer Science, New York.

Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A, Khetani V. (2002). A 90-day oral gavage toxicity study of d-methylphenidate and d, l-methylphenidate in Sprague–Dawley rats. *Toxicology* **179**: 183–196.

Tietze F. (1969). Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal Biochem* **27**: 502–522.

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Vasan RS. (2006). Biomarkers of cardiovascular disease: Molecular basis and practical considerations. *Circulation* **113**: 2335–2362.

Viswanathan MB, Manikandan U, Tangavelou AC. (2005). A new species of *Neurocalyx* (Rubiaceae) from Peninsular India. *Nord J Bot* **23**: 389–394.

Weydert CJ, Cullen JJ. (2009). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nat Protoc* **5**: 51–66.

Wolf PL. (1999). Biochemical diagnosis of liver disease. *Indian J Clin Biochem* **14**: 59–90.

Yao P, Li K, Song F, Zhou S, Sun X, Zhang X, Nussler AK, Liu L. (2007). Heme oxygenase-1 upregulated by ginkgo biloba extract: Potential protection against ethanol-induced oxidative liver damage. *Food Chem Toxicol* **45**: 1333–1342.

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ORIGINAL ARTICLE

The developmental effects of isoflavone aglycone administration on early chick embryos

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ABSTRACT

Soybeans contain the isoflavone aglycone, an endocrine disrupter. To determine the effects of small amounts of isoflavones on developmental processes, we administered 6.25, 62.5, or 625 µg isoflavone per egg to early stage (stage 10) developing chick embryos via the yolk just beneath the embryo. Eggs were kept at 37±0.5 °C and >80% relative humidity, with one rotation per hour for 48 hrs. The embryos were observed under a stereomicroscope for morphological abnormalities and number of somites. Relative to control eggs, there were no significant differences in the average number of somites in eggs administered isoflavone aglycone. Isoflavone, however, had a dose associated effect on abnormal embryogenesis. Embryos treated with isoflavone aglycone showed developmental arrest not reaching somitegenesis, dysmorphology of the neural tube, and shortening of entire embryos.

KEY WORDS: isoflavone aglycone; estrogen receptor; chick embryo; somites; abnormality

Introduction

The Japanese population consumes large quantities of soy products, including tofu, miso, and fermented soybeans, which contain isoflavones. In women, soy isoflavone is associated with reduced incidence of breast cancer and menopausal disorders (Kokubo *et al.*, 2007; Iwasaki *et al.*, 2008; Messina *et al.*, 2006; Nagata, 2010).

Isoflavone is a flavonoid classified as a polyphenol and is present in many leguminous plants, including soybeans. Isoflavone aglycone contains genistein, daidzein, and glycitin, and glycosides include daidzin, glycitin, and genistin. The effects of isoflavone are similar to those of estrogen (Kroon *et al.*, 2004). Components of isoflavone, called phytoestrogens, bind to estrogen receptors and act as an estrogen agonist. Soy isoflavone has been reported effective in reducing menopausal disorders and type 2 diabetes mellitus (Anderson *et al.*, 1999; Jayagopal *et al.*, 2002).

Estrogen has profound effects on menstruation and pregnancy. Following menopause, the secretion of estrogen decreases. This reduction, resulting in a lack of estrogen binding to osteoblasts and an increase in activity of osteoclasts, may frequently lead to the development of osteoporosis in postmenopausal women. Since estrogen acts to regulate the amount of calcium in bone (Popat *et al.*, 2009; Lacroix *et al.*, 2008; Hisa *et al.*, 2008), the phytoestrogen isoflavone may be useful for maintaining the health of postmenopausal women (Mann *et al.*, 2007; Reinwald *et al.*, 2006).

In recent years, the consumption of soybean containing foods has decreased in Japan. Many dietary supplements in Japan include soy isoflavone. It is unclear, however, whether the consumption of isoflavone in dietary supplements is equivalent to its consumption in soy containing foods. Although the biological effects of isoflavone have been studied in Japan, a clear conclusion has not been reached. The Japanese Food Safety Commission recommended an upper limit of soy isoflavone aglycones of 70–75 mg/day (The Japanese Food Safety Commission, 2006) . The effects of these large quantities of isoflavone on embryo development are not known. We have developed a toxic experimental method using early chick embryos (Saito *et al.*, 2012). It thus seems that attention should be paid to the teratogenicity of isoflavone, which is of emphasized merit. We therefore assessed the biological effects of the isoflavone aglycone administration on early chick embryos.

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Materials and methods

The Japanese Food Safety Commission has established an upper limit of soy isoflavone aglycone as 75 mg/day, or 1.25 µg/g. The average weight of a fertilized egg is 50 g, thus the basic quantity of isoflavone administered per egg was 62.5 µg.

Isoflavone aglycones were purchased from Wako Pure Chemical Co. Ltd (Tokyo). Stock solutions of 125 µg/µl were prepared in dimethyl sulfoxide (DMSO) and diluted into phosphate buffered saline (PBS, pH7.0, Wako) to concentrations of 12.5 µg/µl and 1.25 µg/µl.

White Leghorn eggs, fertilized within 24 hrs after laying, were purchased from the Nippon Institute for Biological Science. Twelve eggs were used in the control group or the experimental (isoflavon administered) group. Using a Hamilton syringe, we injected each egg yolk with 5µl isoflavone aglycones (125, 12.5 or 1.25 µg/µl) or DMSO, also diluted in PBS (negative control), through a pin hole made on the shell. Therefore, the amount of isoflavone aglycone administered per embryo was 0, 6.25, 62.5 or 625 µg. It was difficult to define the exact site of administration relative to the embryo, except that it was just underneath the embryo. A hole 2 cm in diameter was cut into each shell to allow to visualize the embryos at stage 10, and sealed with cover silicon gum (KE3475T, Shin-Etsu Chemical Co. Ltd., Tokyo) adhesive with polyethylene film. The eggs were placed in the incubator (model 05, Showa Furanki Ltd.) and incubated for 48 hr at 37±0.5 °C and >80% relative humidity, with one rotation per hr.

The embryos were subsequently excised from the shells and fixed in 70% alcohol for >4 days. The external appearance of each embryo was examined under a stereomicroscope (S8APO, Leica, Germany) and the number of somites was counted. Developmental stage was scored as described (Hamburger *et al.,* 1951).

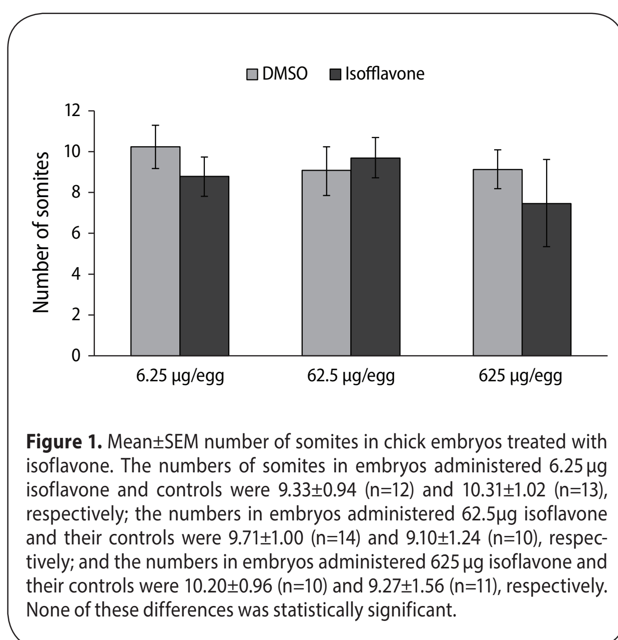

Figure 1. Mean±SEM number of somites in chick embryos treated with isoflavone. The numbers of somites in embryos administered 6.25 µg isoflavone and controls were 9.33±0.94 (n=12) and 10.31±1.02 (n=13), respectively; the numbers in embryos administered 62.5µg isoflavone and their controls were 9.71±1.00 (n=14) and 9.10±1.24 (n=10), respectively; and the numbers in embryos administered 625 µg isoflavone and their controls were 10.20±0.96 (n=10) and 9.27±1.56 (n=11), respectively. None of these differences was statistically significant.

Statistical analysis

The mean numbers of somites in groups of embryos were compared using Student's t test and the incidence of abnormalities was compared using the chi square test. Statistical significance was defined as p <0.01 or p <0.05.

Results

The mean ± SEM number of somites was similar in embryos treated with the 3 concentrations of isoflavone and each set of control embryos (Figure 1). While control chick embryos developed normally (*e.g.* Figure 2), those treated with isoflavone aglycone were malformed, with end neural tube dysmorphology and cylindrical heads (*e.g.* Figure 3). Stasimorphia chick embryos were also observed, with a head and neural tube, but no somite (Figure 4). Other embryos showed head dysplasia and end neural tube dysmorphology (Figure 5). Some embryos showed heteroplasia of the head and neural tube dysmorphology, with an open end of the neural tube (Figure 6) or a plate-shaped head (Figure 7).

Altogether, malformations were observed in 58.3%, 71.4%, and 90.9% of embryos administered 6.25 µg, 62.5 µg and 625 µg isoflavone, respectively (Figure 8). In comparison, malformations were observed in 15.4%, 20%, and 0% of embryos administered 1:10, 1:100, and 1:1000 fold dilutions of DMSO, respectively. The incidence of malformations was significantly higher in each of the isoflavone group than in its control group (p<0.05).

Discussion

The female sex hormones estrogen and progesterone are secreted periodically and maintain various physical functions. Isoflavones are phytoestrogens that interact with estrogen receptors and function as weak estrogens. Genistein binds more strongly to estrogen receptors than daidzein. Imbalances in female sex hormone may result in various disorders.

The Japanese Food Safety Commission has set the upper limit of intake of soy isoflavone aglycones as 70–75 mg/day, the Italian Ministry of Health recommended that isoflavone supplements should not exceed 80mg/day, and the French Food Safety Agency set the quantity of isoflavone aglycone at 1 mg/kg/day (Setchell *et al.,* 1997). The risks of phytoestrogen have also been assessed by the U.S. FDA (Food and Drug Administration) and the AHRQ (Agency for Healthcare Research and Quality).

The effects of soy isoflavone have been assessed in fetuses and babies. The concentrations of plasma genistein and daidzein were significantly higher in 4-month-old babies who consumed 900–1000 ml soybean milk than in 4-month-old babies who consumed cow's milk and breast milk (Xu *et al.,* 1994). The concentration of soy isoflavone is lower in the breast milk of a woman who eats soybean containing foods than in soybean. As yet, however, there are no guidelines related to the safe consumption for

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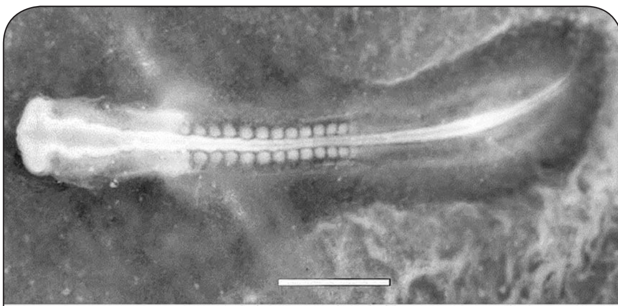

Figure 2. Photograph of a chick embryo administered DMSO (control), showing normal development. A white bar represents 1 mm.

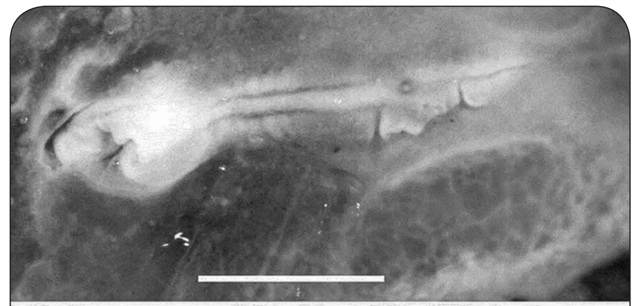

Figure 3. Photograph of a chick embryo administered 6.25 µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

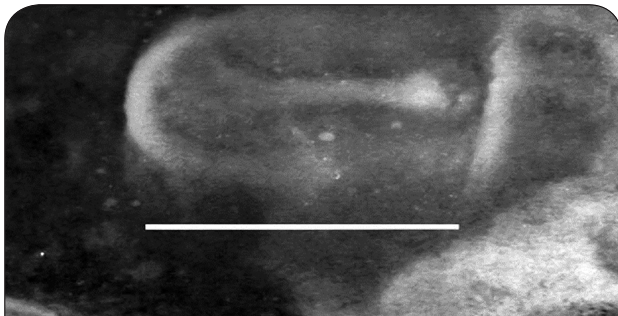

Figure 4. Photograph of a chick embryo administered 625 µg isoflavone aglycone, showing neural tube dysmorphology. A white bar represents 1 mm.

Figure 5. Photograph of a chick embryo administered 625µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

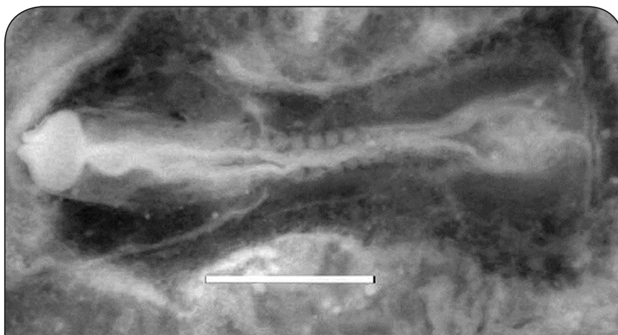

Figure 6. Photograph of a chick embryo administered 625µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

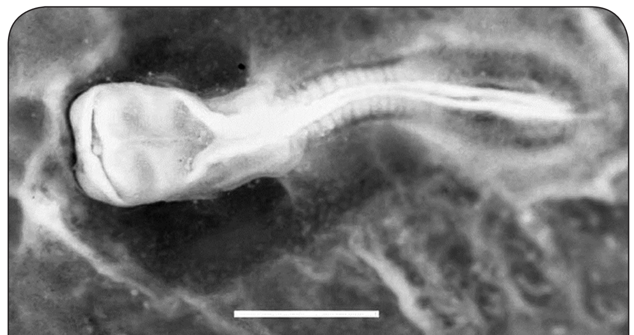

Figure 7. Photograph of a chick embryo administered 625µg isoflavone aglycone, showing heteroplasia of the head and neural tube dysmorphology. A white bar represents 1 mm.

infants of soy isoflavone in soybean milk (Chen *et al.,* 2004). Moreover, the effects of maternal consumption of soy isoflavone on the estrogen synthesis pathway in fetuses have not yet been determined.

Among the soy isoflavone aglycones are genistein, daidzein and glycitin. Genistein treatment resulted in malformation of zebrafish embryos, including spinal kyphosis (Kim *et al.,* 2009), suggesting that excess genistein is a teratogen in exposed zebrafish embryos. Female mice treated neonatally with genistein (50 mg/kg/day) have ovarian follicles and are infertile even after superovulation (Jefferson *et al.,* 2009).

Exposure of pregnant animals to high concentrations of soy isoflavone aglycones may have deleterious effects on fetal health (Newbold *et al.,* 2001; Wisniewski *et al.,* 2003).

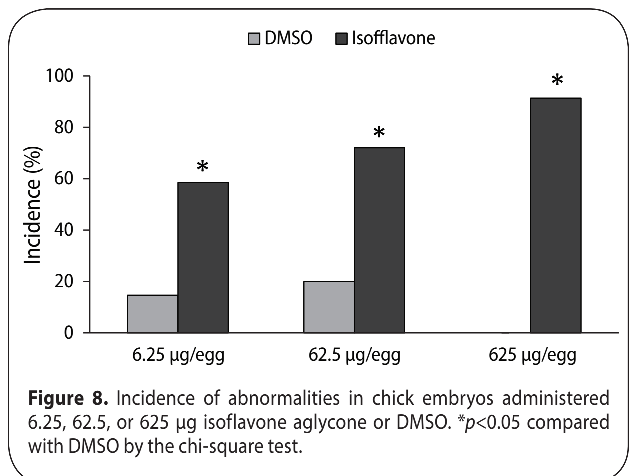

Figure 8. Incidence of abnormalities in chick embryos administered 6.25, 62.5, or 625 µg isoflavone aglycone or DMSO. *$p<0.05$ compared with DMSO by the chi-square test.

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The results of animal experiments cannot be extrapolated to human fetuses. To date, little is known about risks to fetuses from maternal consumption of large amounts of soy isoflavone aglycones.

We found that administration of isoflavone aglycones influenced the development of early stage chick embryos. The incidence of embryo malformation was associated with the dose of isoflavone aglycones.

Conclusions

We observed that exposure of developing chick embryos to soy isoflavone aglycones resulted in adverse effects in a dose-responsive manner. Although the number of somites did not differ significantly between embryos administered isoflavone aglycones and control embryos, the incidence of malformations was significantly higher in groups administered each dose of isoflavone aglycones than in its respective control group. The abnormal embryogenesis associated with soy isoflavone aglycones included dysmorphology of the neural tube, heteroplasia of the head, and plate-shaped heads.

The presented study suggests that it may be desirable to restrict or prohibit administration of isoflavone at least for females in the reproductive generation.

It is eagerly awaited that the clinical etiology of teratogenicity of isoflavone will be elucidated in the future.

REFERENCE

Anderson JW, Smith BM, Washnock CS. (1999). Cardiovascular and renal benefits of dry bean and soybean intake. *Am J Clin Nutr* **70** (Suppl.3): 464–474.

Chen A, Rogan WJ. (2004). Isoflavones in soy infant formula: a review of evidence for endocrine and other activity in infants. *Annu Rev Nutr* **24**: 33–54 (Review).

Hamburger V, Hamilton HL. (1951). A series of normal stages in the development of the chick embryo. *J Morphol* **88**: 49–92.

Hisa I, Kaji H, Inoue Y, Sugimoto T, Chihara K. (2008). Fasting plasma glucose levels are related to bone mineral density in postmenopausal women with primary hyperparathyroidism. *Int J Clin Exp Med* **1**: 319–326.

Iwasaki M, Inoue M, Otani T, Sasazuki S, Kurahashi N, Miura T. (2008). Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: A nested case-control study from the Japan Public Health Center-Based Prospective Study Group. *J Clin Oncology* **26**: 1677–1683.

Jayagopal V, Albertazzi P, Kilpatrick ES, Howarth EM, Jennings PE., Hepburn DA, Atkin SL. (2002). Beneficial effects of soy phytoestrogen intake in postmenopausal women with type 2 diabetes. *Diabetes Care* **25**: 1709–1714.

Jefferson WN, Padilla-Banks E, Goulding EH, Lao SP, Newbold RR, Williams CJ. (2009). Neonatal exposure to genistein disrupts ability of female mouse reproductive tract to support preimplantation embryo development and implantation. *Biol Reprod* **80**: 425–431.

Kim DJ, Seok SH, Baek MW, Lee HY, Na YR, Park SH, Lee HK, Dutta NK, Kawakami K, Park JH. (2009). Developmental toxicity and brain aromatase induction by high genistein concentrations in zebrafish embryos. *Toxicol Mech Methods* **19**: 251–256.

Kokubo Y, Iso H, Ishihara J, Okada K, Inoue M, Tsugane S. (2007). Association of dietary intake of soy, beans, and isoflavones with risk of cerebral and myocardial infarctions in Japanese populations: the Japan Public Health Center-based (JPHC) study cohort I. *Circulation* **116**: 2553–2562.

Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, Williamson G. (2004). How should we assess the effects of exposure to dietary polyphenols *in vitro*?. *Am J Clin Nutr* **80**: 15–21.

LaCroix AZ, Kotchen J, Anderson G, Brzyski R, Cauley JA, Cummings SR, Gass MJ. (2008). Calcium plus vitamin D supplementation and mortality in postmenopausal women: The women's health initiative calcium – vitamin D randomized controlled trial. *Gerontol A Biol Sci Med Sci* **64**: 559–567.

Mann GE, Rowlands DJ, Li FY, de Winter P, Siow RC. (2007). Activation of endothelial nitric oxide synthase by dietary isoflavones: role of NO in Nrf2-mediated antioxidant gene expression. *Cardiovasc Res* **75**: 261–274.

Messina M, McCaskill-Stevens W, Lampe JW. (2006). Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. *J Natl Cancer Inst* **98**: 1275–1284.

Nagata C. (2010). Factors to consider in the association between soy isoflavone intake and breast cancer risk. *J Epidemiol* **20**: 83–89.

Newbold RR, Banks EP, Bullock B, Jefferson WN. (2001). Uterine adenocarcinoma in mice treated neonatally with genistein. *Cancer Res* **61**: 4325–4328.

Popat VB, Calis KA, Vanderhoof VH, Cizza G, Reynolds JC, Sebring N, Troendle JF, Nelson LM. (2009). Bone mineral density in estrogen-deficient young women. *J Clin Endocrinol Metab* **94**: 2277–2283.

Reinwald S, Weaver CM. (2006). Soy isoflavones and bone health: a double-edged sword?. *J Nat Prod* **69**: 450–459.

Saito K, Niijima A, Kamite E, Watanabe M. (2012). Bisphenol A and estrone-induced developmental effects in early chick embryos. *Environ Toxicol* **27**: 58–62.

Setchell KD, Zimmer-Nechemias L, Cai J, Heubi JE. (1997). Exposure of infants to phyto-oestrogens from soy-based infant formula. *Lancet* **350**: 23–27.

The Japanese Food Safety Commission. (2006). Fundamental Concepts in the Safety Assessment of Foods Containing (Soy Isoflavones for the purpose of Specified Health Use). Novel Foods Expert Committee Attached Sheet. 40–41.

Wisniewski AB, Klein SL, Lakshmanan Y, Gearhart JP. (2003). Exposure to genistein during gestation and lactation demasculinizes the reproductive system in rats. *J Urol* **169**: 1582–1586.

Xu X, Wang HJ, Murphy PA, Cook L, Hendrich S. (1994). Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J Nutr* **124**: 825–832.

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