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GENOTOXIC AND CYTOTOXIC EFFECTS OF ALKALOIDS EXTRACTED FROM *RUBIA CORDIFOLIA* ROOTS ON MICE BONE MARROW CELLS

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Abstract

In the present study, *Rubia cordifolia* alkaloids were investigated for their effects on the chromosomal aberrations and mitotic activity of Swiss mice bone marrow cells. Different concentrations of *R. cordifolia* were used at different periods of time. It was found that *R. cordifolia* alkaloids induce a significant numbers of aberrations. The frequencies of aberrations were found to be dose and time dependent. On the other hand, a remarkable decrease in the mitotic index was found at all periods of exposure and most of the applied concentrations compared to the control. Furthermore; cytotoxic effects, measured using MTT assay, showed that the proliferation index increases as the concentration and time of the experiment increases. Our results suggest that *R. cordifolia* alkaloids have a great potential of anti-proliferative activity, weak chromosome-damaging effects at high doses and long duration. Thus *R. cordifolia* alkaloids can be considered safe at low doses.

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Key words*: Rubia cordifolia* , cytotoxicity, genotoxicity , MTT assay, Alkaloids

Introduction

Nature has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources [1]. The plant-based traditional medicine systems continues to play an essential role in health care, about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care [2]. Toxicity testing can detect some of herbs risks that may be associated with various uses, therefore avoiding potential harmful effects when used as medicine [3]. There are several groups of herbs that may induce harmful effect in patients. First, herbs that contain near pharmaceutical concentrations of poisonous constituents. For an examples Atropa belladonna, Arnica spp, Aconitum spp, and Digitalis spp [4]. Second, herbs with very powerful actions, often causing,nausea or vomiting. Examples include Lobelia and Eonymus spp. The hepatotoxicity of pyrrolizidine-alkaloid-containing plants such as Comfrey (Symphytum) is the best known among them. Dryopteris (Male Fern), Viscum (Mistletoe) and Corynanthe (Yohimbe) can be quoted as other examples. Given the prevalence of side effect associated with those herbs, laymen are advised to avoid internal consumption [5].

In the last decade, the interest in plant extracts studies and their biologically active compounds has increased, thus natural therapies or herbal medicine were used by the pharmaceutical industry [6]. Genotoxicity is a special area in toxicity testing, and it is often considered the most difficult to detect. It may be defined as a chemically induced mutation or alteration of the structure and/or segregation of genetic material [7]. There are two broad classes of chromosomal aberrations. First, numerical aberrations where the whole chromosomes are either lost or added. Second, structural aberrations, where major changes in the structure of the DNA happen due to breakages, deletions, exchanges, or rearrangements of DNA [8]. The MTT 3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay is the most frequent method used for measuring cell proliferation and cytotoxicity [9]. The MTT assay works by the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure t in vitro cytotoxic effects of drugs on cell lines or primary patient cells [10].

Alkaloids are considered as a notable class of defense compounds, forming the largest group among the nitrogen-containing secondary metabolites. They were known to humans for several centuries; the first identified alkaloid was morphine, which was extracted from opium poppy.Over 12,000 alkaloids have been identified [11], which have a range of features from beneficial to lethal effects on humans [12]. The traditional therapeutic use of R. cordifoliahas been for skin disorders, anticancer activity, anti-inflammatory, urinary disorders, anti-stress, antimicrobial, hepatoprotective, radio protective, hypotensive, analgesic, antimalarial, antileukemic, immunomodulatory and antioxidant [13,14]. Also, other pharmacological actions like blood purifier activity, astringent, antidysentric, antiseptic, antirheumatic, anti-tumor, and antihyperglycemic were also reported [15]. Apart from its medicinal value, this plant has been used as natural food colorant and as natural hair dyes. The interest in the isolation of natural dyes and coloring matters is increasing due to their applications in food, drugs and other human consumptions [16].

R. cordifoliawas continuously screened in laboratory for its pharmacological properties. An extensive phytochemical investigation realized the presenceof various secondary metabolites, such as indole alkaloids, cardiac glycosides, tannins, flavonoids, terpenoids, anthraquinone and phenols [17,18]. Literature review on the phytochemical constituents of R. cordifolia revealed that alkaloids are its major components [19].

On the basis of the wide use of the plant in the folkloric medicine,this study aimed to investigate the genotoxic and cytotoxic effects of alkaloids, extracted from R. cordifolia roots.Understanding such effects could possibly enhance the useof Rubiaclinically with minimal toxic effects.

Methods

Preparation of the extract

About 500 g of dried roots of R. cordifolia were cut into small pieces then grinded to a moderately coarse powder and packed loosely in a porous cellulose thimble Whatman number 4, which was immersed completely in ethanol in a soxhlet extractor. The concentrated ethanolic extract was mixed with 10% HCl, then chloroform was added to extract the aqueous acidic layer and adjust the pH to 9.5. The chloroform layer was evaporated under vacuum at 45-50ºC to give the alkaloidal matter. Mayer's Reagent precipitation test was performed to confirm the presence of alkaloids. The alkaloidal fraction was then suspended in Dimethyl Sulphaoxide DMSO until forming homogenous

solution which was considered as a stock solution, with concentration of 560 (mg/kg) body weight.
A group of concentrations of this ex-tract were prepared. The concentrations of (140 mg/kg, 280 mg/kg, 420 mg/kg, and 560 mg/kg were applied to investigate the genotoxic and cytotoxic effects of different concentrations of R. cordifolia alkaloids on bone marrow cellsfor different time intervals: 24, 48 and 72h.For each dose, five male mice were used for every experimental and control group. Control groups were given equal volumes of DMSO.

Preparation of mouse bone marrow cells

Swiss albino mice JVC 1 of 10-12 weeks old were sacrificed by cervical dislocation, after a single intraperi-toneal injection. Femora were carefully cleaned from adherent tissues. The tip of each bone was removed and the bone marrow was harvested using sterile saline [20,21]. DMSO served as a control [22]. Metaphase bone marrow cells were prepared for chromosomal aberrations and mitotic investigation by the classical methods. The preparations were stained with Giemsa solution. Slides were coded and scored for the presence of dividing cells. 2000-4000 cells were scored. Frequency of normal and structurally aberrant chromosomes were examined, and chromosomal aberrationwas calculated according to the following formula [23,24].

Chromosomal aberration(CA) (%) = (Total number of chromosomal aberrations/ Total number of cells examined)*100

While the mitotic index was calculated according to the following equation [25].

Mitotic index =(Number of divided cells/Total number of cells)*100

Viability Test of bone marrow Cells

After bone marrow cells were harvested, a fresh medium RPMI-1640 (HEPES, 10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin) was incubated in a 37°C water bath for 30 min then added to the cells and centrifuged at 1200 rpm for 8 min. The cell pellet was re-suspended in 1 ml of the medium. Initial cells viability and counting was determined using direct Trypan-blue (Sigma, USA) exclusion test using hematocytometer.

Cell culture

Isolated bone marrow cells, at a density of 1.0 x106 cells /ml was prepared in a final volume of 10 ml culture medium and placed in 25 cm3 tissue culture flasks. Concentrations of alkaloids of 17.5 mg/ml, 35 mg/ml, 52.5 mg/ml and 70 mg/ml were added to the culture and incubated at different time intervals 24, 48 and 72 hours. DMSO served as a control.

Cell Proliferation Assays (MTT assays)

Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) MTT assay. MTT was dissolved in PBS at 5 mg/ml (Bioworld, USA) at each time interval according to Mosmann study (1998) [26]. Briefly the cultured cells at a density of 50 x 103cells/ml were mixed with 40µl MTT in 24-well plate for each well, a triplicate for each dose level was done for each of the time intervals and further incubated at 37°C in a humidified atmosphere containing 5% CO2 for four hours. After the incubation period, 40 µl of homogenous solution (DMSO: Isopropanol) (TEDIA, USA) was added to the mixture to dissolve the dark blue crystals. The absorbance (O.D) at 570 nm was measured 15 min later on ELISA microplate reader. Proliferation index (PI) was calculated according to this equation:

PI= (O.D. of treated culture/ O. D. of untreated culture) x 100 .

Statistical analysis

Statistical analysis between the treated groups was analyzed by ANOVA and Bonferroni post-tests, using the GraphPad Prism 5 software. P values less than 0.05 were considered to be statistically significant. Standard error of the mean (SEM) was also calculated.

Results

Effects of R. cordifoliaAlkaloids on the Formation of Chromosomal Aberration

Genotoxic affects of *R. cordifolia* alkaloids were studied on mice bone marrow cells. Different concentrations of *R. cordifolia* alkaloids (140, 280, 420 and 560 mg/kg) with four different periods of time (8, 24, 48 and 72 h) were applied. Through this study, the capacity of *R. cordifolia* alkaloids to induce chromosomal aberration on mice was noticed after scanning (2000-4000) cells per treatment.
Statistical analysis of the data revealed that there is a significant difference ($p \leq 0.05$) between the control and treated groups with 560 mg/kg group and after 72 h exposure. On the other hand there are no significant differences ($p > 0.05$) between the other treatments and the control. Thus the chromosomal aberration percentages increased in most cases with

concentrations increase as well as with increasing exposure times (Figure 1)

Effects of R. cordifolia Alkaloids on the mitotic index of Mice bone marrow cells

Genotoxic effect of *R. cordifolia* alkaloids was studied on the basis of the mitotic index (MI). In this study (MI) was calculated for bone marrow cells of Swiss albino mice exposed to different concentrations of *R. cordifolia* alkaloids (140, 280, 420 and 560 mg/kg) with different periods of time (8, 24, 48 and 72 h). The rate of cell division demonstrates dose and time dependence as shown in (Figure 2 A-D). A notable decrease in the mitotic index was evident in the bone marrow cells at all concentrations and in all four periods of time when compared with the control.

The rate of cell division demonstrates dose and time dependence as shown in (Figure 2 A-D). A notable decrease in the mitotic index was evident in the bone marrow cells at all concentrations and in all four periods of time when compared with the control. The statistical analysis of the data revealed that there is a significant difference (P≤ 0.05) between the treated groups of (280, 420 and 560 mg/kg) and the control at 48 and 72h, while the lowest concentration (140 mg/kg) of *R. cordifolia* alkaloids shows no significant differences between treated groups and control at 8, 24 and 48 hours. Nevertheless, it was significantly different at 72 hours. Eventually; as the dose increases there was a significant decrease in the mitotic index.

Effects of R. cordifolia Alkaloids on Proliferation of Mice Bone Marrow Cells

MTT assay has long been utilized to quantify cellular capacity. In this study it is clear that *R. cordifolia* alkaloids enhance cell killing when different concentrations (17.5, 35, 52.5, 70 mg/ml) were applied at different time intervals (24, 48, 72 h) on cells. These results indicate that treatment with *R. cordifolia* alkaloids caused a remarkable increase in the proliferation index compared to untreated controls. As the concentrations and time intervals increase, the proliferation inductive potential of cells increased then decreased at 72 hours of exposure compared to other periods of times. Therefore *R. cordifolia* alkaloids induced the highest effect at 48 hours of exposure. Moreover, the statistical analysis of the data revealed that there is a significant difference (P≤ 0.05) between the treatment groups and the control in all treatments (DMSO) (Figure 3).

Discussion

The response of cells to cytotoxic agents depends on their sensitivity to the concentrations and the exposure times of these agents [27]. In this study, cytogenetic effects of R. cordifolia were measured by two parameters; chromosomal aberrations and the mitotic index, while cytotoxic effect was measured by the proliferation index using MTT assay. Chromosomal aberration (CA) assays are considered to be sensitive assay that recognize the genotoxic effects induced by chemicals (WHO, 2001). Our results showed that R. cordifolia alkaloids induced chromosomal aberrations, and the percentage of these aberrations increased by increasing the exposure time (Figure 1).Results presented in this study are in agreement with previously reported assessments [28,29]. Our results indicated that the percentage of chromosomal aberrations increased not only by increasing the time of exposure, but also by increasing the dose. Chromosomal aberrations induced by higher doses are more than those induced by the lower doses, and a significant increase was noticed after 72 hours exposure at the highest dose (560 mg/kg) of R. cordifolia alkaloids. Similar results were obtained by other studies [30,31]. AL-Zubairiet al. (2008) [31] found that chromosomal aberrations showed significant increase in treated group with Catha edulis (Khat) compared to the control.

Alkaloidal fraction may contain alkylating compounds (S-dependent agents) that produce aberrations via misreplication; thus DNA damage occurs when a DNA molecule with lesions undergoes DNA replication [32]. The previous suggestion is supported by the knowledge that plants belonging to the Astraceae family, which contain a variety of alkaloids produce genotoxicity through DNA binding, DNA cross-linking, DNA-protein cross-linking, mutagenicity and carcinogenicity [28]. Furthermore, a delay of chromosomes movement happens when the viscosity of the cytoplasm increases [33,34]. Also these findings are in line with Al-Meshal (1987) study [35], which found the active constituent of khat produced aberrations in the chromosomes. Similar increase reported by Abderrhman in studding cytogenetic effects of *Peganum harmala* extract on maize root tips [36]. The mitotic index (MI) is another parameter measuring the genotoxic effects, which quantify differences in cell division when an environmental parameter is changed [37]. In this study we evaluated *R. cordifolia* alkaloids effects on the division of mice bone marrow cells. A marked decrease in the mitotic index was noticed in all times of exposure and with almost all concentrations

compared with corresponding control, indicating a dose and time dependence. Our results were in concordance with previous studies [38,39]. For example, Abderrahman (2004) found that *R. cordifolia* extract caused a mitotic depression in bone marrow cells of mice [38].

Decreasing of mitotic index can be explained by the arrest of the division of the interphasic nucleus, hindering the onset of prophase and thus the division of the cells [40]. The results showed that R. cordifolia alkaloids are strong mitotic inhibitors and could give rise to mitotic abnormalities with increased concentrations, and have an inhibitory effect. The present study revealed that *R. cordifolia* alkaloids significantly induced MTT reduction at all concentrations after 24, 48, and 72 hrs of exposure. Therefore MTT reduction correlates well with doses and exposure time. We can postulate that higher concentrations produce inhibitory effects on cell growth. Our results were in line with Judith et al. (2009) [36], who reported harmine as a potent inducer of cytotoxicity through necrosis and apoptosis, and a useful inhibitor of tumor development. These findings agree with those obtained by Erhan et al.(2009) [41], who reported that Senecio trapezuntinus as an antiproliferative and antimitotic agent in human lymphocytes.

In Conclusion, our results indicated that R. cordifolia produced chromosomal aberration. Further, genotoxic effects and antiproliferative effects were more prominent at high doses, suggest that alkaloids extracted from R. cordifolia exhibit genotoxic properties as well as antimitotic and antiproliferative activities. Detailed and large scale investigations are required to study the cytogenetic and cytotoxic effects of alkaloids from R. cordifolia using other parameters such as: single strand breaks and comet assay. Further studies are required to determine the effects of different alkaloids isolated from R. cordifoliato evaluate the synergistic effects on MI, PI and CA and other parameters.

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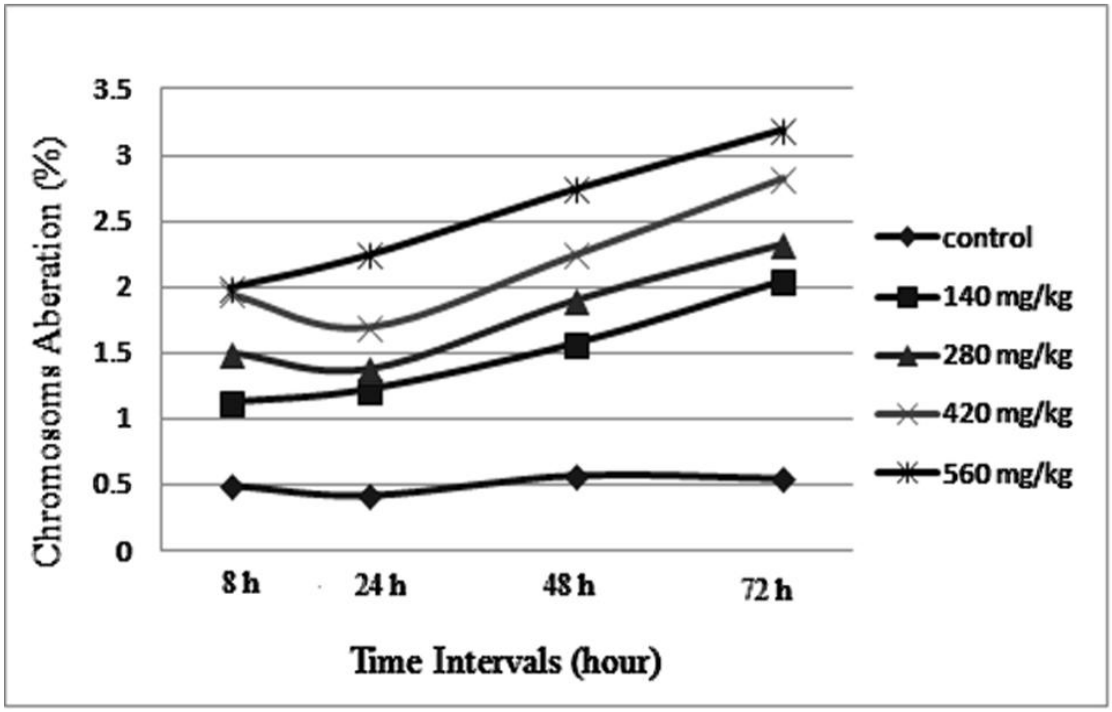

Figure 1. Effect of R. cordifolia alkaloids on the formation of chromosomal aberrations on mice cells. Control= 10 µl DMSO. (source:MS-Office/PC)

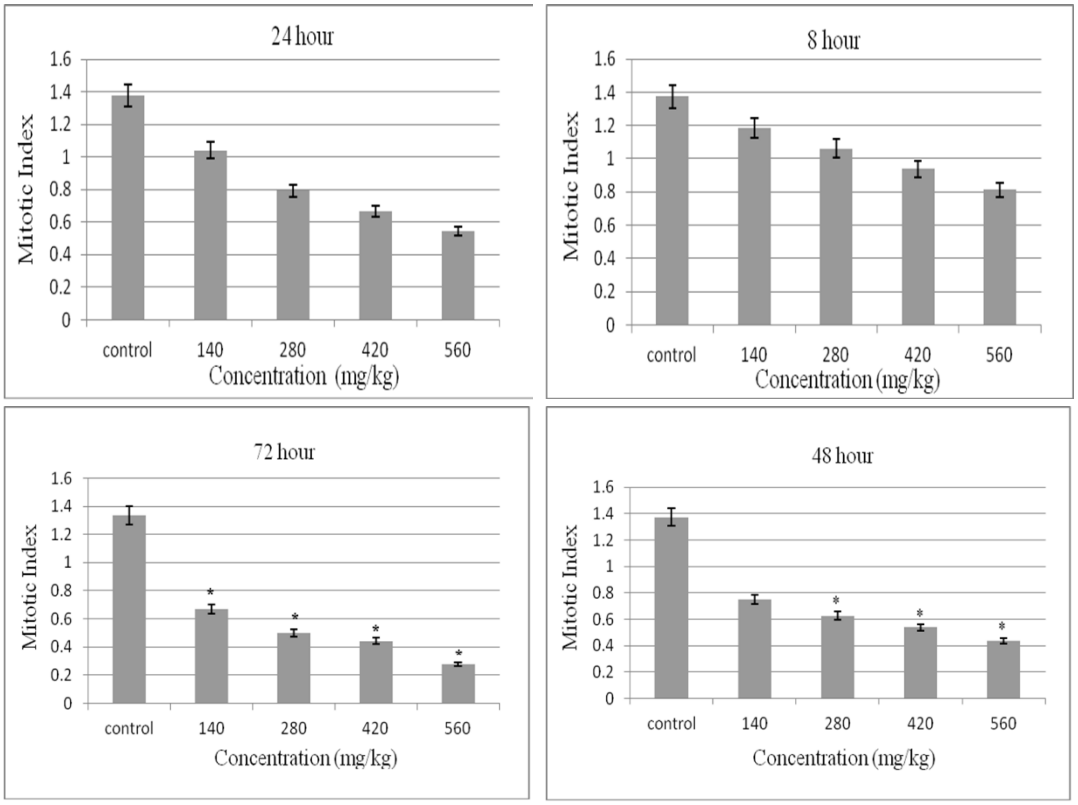

Figure 2. Effects of R. cordifolia alkaloids on Mice Cell Division. (A) treatment at 8 hours, (B) 24 hours, (C) 48 hours, (D) 72 hours. The error bars refer to standard errors of the means. Control mice were treated with 10 µl DMSO..
* refers to significance between control and treated mice at P ≤ 0.05. (source:MS-Office/PC)

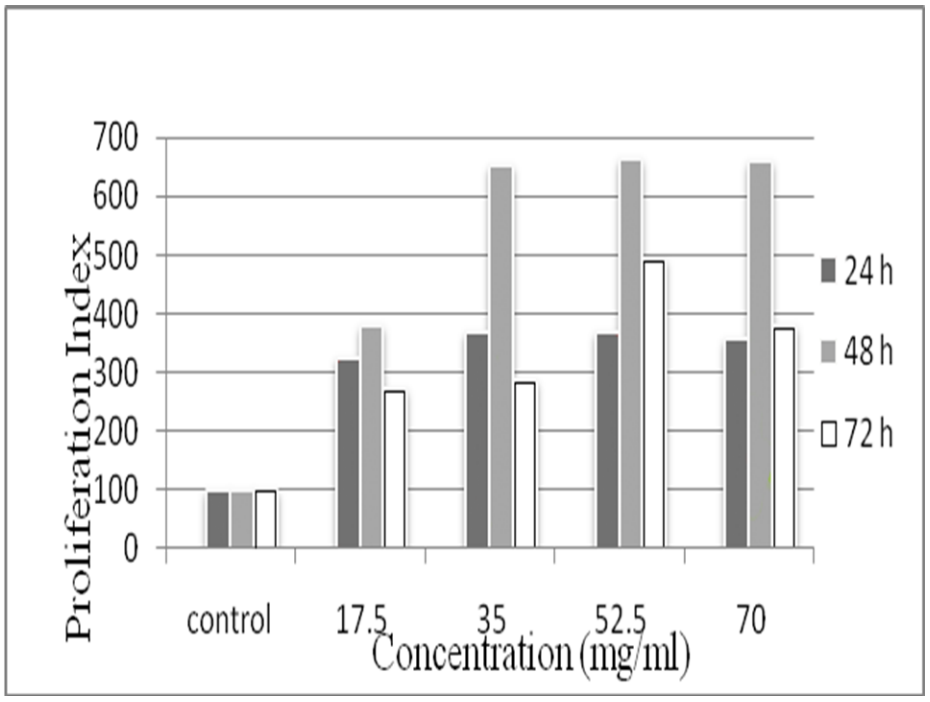

Figure 3. Effect of R. cordifolia alkaloids on proliferation index for different concentrations (17.5 mg/ml, 35 mg/ml, 52.5 mg/ml, and 70 mg/ml) at different periods of exposure (24 hrs, 48 hrs, and 72 hrs). (source:MS-Office/PC)