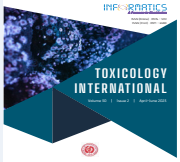
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Genotoxicity of Beta-Sitosterol, Betulinic Acid, Biochanin A, and Ursolic Acid, Potential Anthelmintic Phyto-Compounds

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Abstract

Beta-sitosterol, betulinic acid, biochanin A and ursolic acid are known to possess anthelmintic properties. This study was undertaken to assess the genotoxic potentials of these phytocompounds in albino rats. Animals were orally administered with 1 and 10 mg/ml of the above phyto-compounds for a period of 14 days. All the animals were subjected to mitotic arrest before sacrifice and then bone marrow cells were collected from both the femurs and processed to observe the chromosomal aberrations. The results revealed that ursolic acid induced the maximum number of statistically significant abnormalities ($p \leq 0.05$) followed by betulinic acid and biochanin A. Beta-sitosterol was found to be the safest of all the tested phytocompounds as far as chromosomal abnormalities in short term oral studies are concerned as it did not lead to major changes at genomic level. Although these phyto-compounds are considered potential anthelmintics, their long-term use may cause genotoxic effects to the users.

Keywords: Beta Sitosterol, Betulinic Acid, Biochanin A, Genotoxicity, Ursolic Acid

1. Introduction

Several phytocompounds are known to possess therapeutic properties[1]. Despite their beneficial effects, in some cases, they also possess toxic potentials[2]. For any compound to be accepted into the mainstream pharmacopoeia, efficacy and toxicity profiling is necessary. Genotoxicity studies evaluate the genotoxic potentials of test agents by assessing mutations, damage to DNA, chromosomal damage, abnormal recombination, and numerical chromosome changes. Such safety studies are a background for assessment of the safety of new drugs[3].

Phytocompounds such as coumestrol, genistein, biochanin A, and bisphenol A have been reported to cause different types of mutations[4]. Similarly, salsoninol, a tetrahydroisoquinoline compound found in a variety of foodstuffs, including soy sauce, beer and bananas is genotoxic or carcinogenic in cultured Chinese hamster lung fibroblasts which exhibited increased frequencies of chromosomal aberrations[5]. In another study, *Rhazya stricta* an alkaloid, revealed major signs of genotoxicity and clastogenicity in rat leukocytes[6]. Also, aristolochic acid, a bioactive constituent known for its anti-snake venom, abortifacient, antimicrobial, and antioxidant properties was found to be genotoxic in the *Allium cepa* assay[7]. Accordingly, several other medicinal plants have been shown to possess genotoxic potentials[8,9]. In contrast, some compounds have also been found to be lacking genotoxic potentials[10].

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Betulinic Acid (BA) is a pentacyclic triterpene acid present in plants belonging to the family Betulaceae and is also now known to be present in other plant families as well. It is known to possess cytotoxic, anti-HIV, anti-inflammatory, antidiabetic, antimicrobial, antiprotozoal,[11] and anti-cancer properties[12]. Ursolic Acid (UA), is a pentacyclic terpenoid carboxylic acid and is known to possess antioxidative, anti-inflammatory, anticancer, and hepatoprotective activities[13]. It is present in several vegetables, fruits and medicinal plants[14]. Biochanin A (BCA) is an isoflavone found in several plants such as red clover, cabbage, chickpea, and herbal dietary supplements. It is known to possess antihyperglycemic, antioxidative, anti-tumour, anticholinergic, neuron protective[15], and anti-bacterial properties[16]. Beta-Sitosterol (BS) is a dietary phytosterol with numerous medicinal properties[17] including anthelmintic[18] and anti-cancer activity[19].

All these phyto-compounds have also been known to be potent anthelmintics[18]. This study was conducted to evaluate the genotoxic potential of beta-sitosterol, betulinic acid, biochanin A, and ursolic acid, in Wistar rats using a chromosomal aberration assay.

2. Materials and Methods

2.1 Experimental Animals

Wistar rats of either sex, about 8 weeks old and weighing about 180-200 g were used for the study. Animals were maintained in separate cages and before the experiment they were acclimatised to laboratory conditions. Animals were exposed to 12 h light and 12 h dark cycles. They also had ad libitum access to food and water[20]. Experiment on animals was duly approved by the Institutional Ethics Committee (IEC) (Animal Models) of North-Eastern Hill University (NEHU) (Vide, Member Secretary, IEC, NEHU, dated December 4, 2014, approval number 01). All experiments were conducted strictly as per the guidelines laid by the IEC and ARRIVE guidelines.

2.2 Chromosomal Aberration Assay

Chromosomal aberrations were studied to ascertain the possible genotoxic effects in vivo on Wistar rats by using Chromosomal Aberration (CA) assay as described by Dolan *et al.*[21] and Sankar *et al.*[22]. The lowest and highest tested doses of phytocompounds were selected exponentially and by the doses used in in vivo anthelmintic and sub-acute toxicity studies as per OECD guidelines[20]. Animals were divided randomly into three groups, comprising 3 animals in each group (n = 3). Dimethyl Sulphoxide (DMSO) was used to dissolve the compounds, hence, control animals (Group I) were fed only the vehicle (water) along with a few drops of 1% Dimethyl Sulphoxide (DMSO), whereas, animals in Group II and III received an oral dose of the lowest (1 mg/ml) and highest (10 mg/ml) test doses of phytocompounds, once daily for 14 days, respectively. At the end of the treatment period, all animals were subjected to mitotic arrest by intra-peritoneal injection of colchicine at a concentration of 4 mg/kg body weight (b.w.). Following colchicine injection, 2 h later, all the animals were sacrificed and a maximum of 100 well-spread metaphase spreads were analysed per animal for grading various kinds of chromosomal aberration, which included cytogenetic damage (chromosomal breaks, fragments, deletions, exchanges, and disintegrations), gaps, and polyploidy as described by Dolan *et al.*[23] and Zong *et al.*[24].

2.3 Statistical Analysis

All the experimental data are represented as mean ± Standard Error of Mean (SEM). The p-value ≤ 0.05 were considered statistically significant. All the statistical calculations were done using Origin Pro 2018 SR1.

3. Results

The oral administration of 10 mg/kg b.w. of betulinic acid to Wistar rats revealed significant ($p<0.05$) abnormalities viz., isochromatic breaks, chromatid breaks and chromosomal fragments; thus, revealing upto 8.60±0.74 % of aberrant metaphases in animals after 14 days of treatment with betulinic acid. Likewise, similar effects were seen with 10 mg/kg b.w. ursolic acid-treated animals, where abnormalities including chromatid breaks, isochromatid breaks and chromosomal fragments were recorded. Herein, the mitotic index was recorded to be 5.42±0.34 and the percentage of aberrant chromosomes was found to be 11.80±2.15% which was the highest among the four phytocompounds (Table 1). Oral inoculation of biochanin A at a dose of 10 mg/kg b.w. to rats revealed 7.60±0.87% of aberrant metaphases and the majority of the aberrations comprised of chromatid breaks and chromosomal fragments (Figure 1). Likewise, the oral administration of 10 mg/kg b.w. beta-sitosterol displayed 5.60±1.36% of aberrant metaphases and aberrations such as chromosomal fragments, isochromatid breaks and chromatid breaks. It

Table 1. Frequency of occurrence of different types of chromosomal aberrations, and mitotic index in bone marrow cells of rats treated with different test compounds

Treatment	Total metaphases/ number of mice	Total aberrations	Percentage of aberrant metaphases (mean ± SEM)	Aberration type			Mitotic index (percentage ± SEM)
				CB	ICB	CF	
Control	500/5	9	1.8±0.48	3	0	6	7.42±0.33
BA	500/5	43	8.6±0.74*	11	3	29	6.21±0.21
BCA	500/5	38	7.6±0.87*	10	0	28	6.45±0.45
UA	500/5	59	7.6±0.87*	18	6	37	5.42±0.34
BET	500/5	28	5.6±1.36*	7	4	17	7.02±0.62

CB – Chromatid Breaks; ICB – Isochromatid Breaks; CF – Chromosomal Fragments. Data are expressed as mean ± SEM (n = 5), BA– Betulinic Acid, BCA – Biochanin A, UA – Ursolic Acid, BET – Beta Sitosterol. One way ANOVA *p<0.05 vs control group.

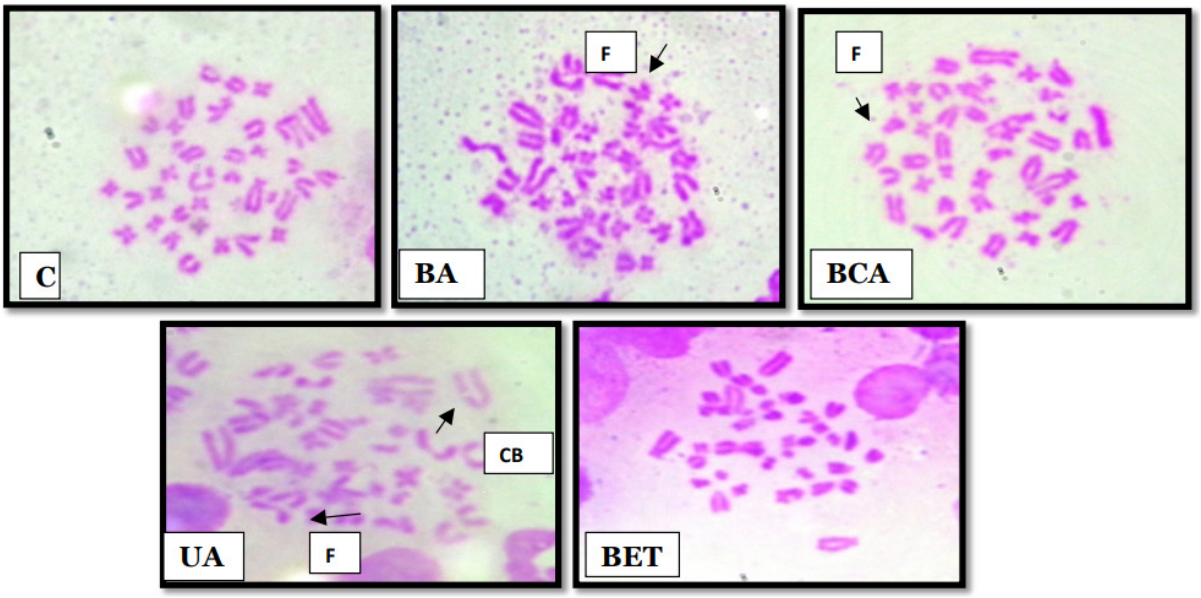


Figure 1. Photomicrographs of bone marrow metaphase chromosome spreads of rats. C - normal set of Chromosomes, BA – Betulinic Acid, BCA – Biochanin A, UA – Ursolic Acid BET – Betulinic Acid (F – chromosome fragments, CB – Chromosome Breaks, 100x).

is noteworthy that isochromatid breaks were not present in biochanin A- treated animals, as observed for the other three test compounds. In brief, ursolic acid induced the maximum number of statistically significant abnormalities ($p \leq 0.05$) followed by betulinic acid and biochanin A. Beta-sitosterol can be considered the safest of all the test phytocompounds as far as chromosomal abnormalities in short term oral studies are concerned as it did not lead to major changes at the genomic level.

4. Discussion

Genotoxicity has been one of the principal parameters used to determine the toxic effects of test agents at the chromosomal level[3]. The present study revealed genotoxic effects in the form of chromatid breaks, isochromatid break and chromosomal fragments. The maximum frequency of various aberrations was seen in the group of animals that were treated with 10 mg/kg b.w. dose of

ursolic acid in the form of chromosomal fragments chromatid breaks and isochromatid breaks. In context with the genotoxicity of biochanin A, the genotoxic effect gets enhanced in the presence of rat hormones which increases the carcinogenicity as revealed by the studies with other estrogenic flavonoids[25]. Similar results have been observed by other workers. A study on oleanolic acid, a naturally occurring pentacyclic triterpenoid which possesses numerous activities revealed it possesses cytotoxic properties[26]. Similarly, eugenol isolated from *Cinnamomum zeylanicum* is genotoxic[27]. Likewise, Hadi *et al.,* also reported that pyrrolizidine alkaloids found in plants as secondary metabolites can cause genotoxicity[28].

It is noteworthy that there are also reports where compounds have been found to possess anti-genotoxic properties. The flavonoids rich root extract of *C. auriculata* has been reported to prevent cyclophosphamide-induced chromosomal aberration, thus indicating its significant anti-mutagenic potential[29]. Also, interestingly, hydroxytyrosol has been tested for its chromosomal aberration potentials in mice bone marrow cells at 2000 mg/kg b.w in vivo. It was seen that hydroxytyrosol did not lead to increased frequency in the number of aberrant cells or any reduction in mitotic index post 24h, thus confirming the ordeal that hydroxytyrosol is not a clastogen[21]. Another phyto-compound genistein was evaluated for its genotoxic potential in rats. During in vitro *Salmonella typhimurium* assay, genistein did not reveal any mutagenic effect, while in the in vitro mouse lymphoma assay, genistein led to a marked increase in the number of resistant mutants, thus depicting genistein to be a potential clastogen[30]. Similarly, the antigenotoxic potential of lupeol was evaluated at a single concentration of 100 mg/kg b.w. and the bone marrow was analysed for chromosomal aberrations. In this study, a decrease in the number of aberrant chromosomes as well as a significant enhancement of mitotic index was seen which is an indication of the cytoprotective abilities of lupeol[31]. In agreement with the findings of the present study, Fujita *et al.,* also reported that ursolic acid can also lead to the induction of chromosomal breaks[32]. The chromosomal aberration study of betulinic acid, biochanin A, and beta-sitosterol has been reported for the first time in this study.

5. Conclusions

The results of the present study indicate that the long-term use of betulinic acid, biochanin A and ursolic acid in higher doses may induce some genotoxic effects in experimental animals. The maximum genotoxic effects were seen for ursolic acid, followed by betulinic acid and biochanin A, and the least effects were observed for beta sitosterol.

6. Acknowledgements

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7. Authors' Contribution

Arun K. Yadav conceptualised and supervised the study, analysed the results, and edited the draft. Vijaya Mishra performed the experiments, analysed the data, and wrote the first draft. Amar Deep Soren assisted in the experiments, analysed data, and wrote the second draft. The final draft was read and approved by all the authors.

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