

***In Vivo* Evaluation of Genotoxic Effect of *Caralluma acutangula* (Decne.) N.E.Br. extract on Mice Bone Marrow Cells**

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ABSTRACT

The current study was designed to evaluate the genotoxicity of *Caralluma acutangula* (Decne.) N.E.Br. extract on mice bone marrow cells *in vivo*. Two cytogenetics parameters were used, chromosomal aberrations assay and micronucleus test. Three different concentrations 125, 250, and 500 mg/kg body weight were chosen. Our results demonstrated that administration of *C. acutangula* extract did not significantly increase the number of structural chromosomal aberrations. No significant differences in the percentages of mitotic indices were observed at the three different concentrations of *C. acutangula* extract. In micronucleus test, treatment with *C. acutangula* extract caused no significant increase in the number of polychromatic erythrocytes with micronuclei or in the mean percentage of polychromatic. In conclusion, *C. acutangula* extract showed no significant genotoxic effect on mice bone marrow cells *in vivo*.

Keywords: *Caralluma acutangula*, genotoxicity, clastogenicity, micronuclei, chromosomal aberrations.

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INTRODUCTION

Traditional medicine has remained the most affordable and easily accessible treatment in the primary healthcare system in rural and remote areas around the world, and local treatment is the only means of treatment medicine for such communities. In Africa, Asia, Latin America and the Middle East, 70-95 percent of the population still use traditional medicine (TM) for primary healthcare. And some 100 million people are believed to use traditional, complementary or herbal medicine in the European Union (EU) alone - as high as 90 percent of the population in some countries (Robinson and Zhang, 2011). Assessment of the potential genotoxicity of traditional medicines is a critical issue as damage to the genetic material may lead to critical mutations and therefore also to an increased risk of cancer and other diseases. Genetic toxicology studies have given rise to many testing procedures, both *in vitro* and *in vivo*. These procedures have been designed to assess the effects of various test substances on the genetic material and consequently, to assess the risk to living organisms including humans (Attia, 2011). Testing *in vivo* on rodents have been used because the metabolic activation and detoxification of the chemicals in the animals are closer to human system. The micronucleus test detects clastogenicity due to chromosome breakage (Attia, 2007), and aneugenicity due to chromosome lagging resulting from imbalances in the mitotic apparatus (Rojas *et al.*, 1993). Alterations in cells such as chromosomal aberrations (CAs)

and sister chromatid exchanges (SCE) have long been applied in surveillance of human genotoxic exposure and early effects of genotoxic carcinogens. The use of these biomarker assays is based on the fact that most established human carcinogens are genotoxic in short-term tests and capable of inducing chromosomal damage. On the other hand, chromosomal aberrations have been used as important cytogenetic biomarkers to study the mutagenic effects of different chemicals *in vivo* and *in vitro*. The relevance of CAs as a biomarker has been further emphasized by epidemiological studies suggesting that a high frequency of chromosomal aberrations is predictive of an increased risk of cancer (Sutiakova *et al.*, 2012). On the other hand, SCE is a highly sensitive parameter for evaluating human occupational and environmental exposure to mutagenic and carcinogenic agents. Moreover, parameters such as Mitotic Index (MI) and Cell Proliferation kinetics (CPK) have been proposed as useful biomarkers for the pre-screening of the potential cytostatic activity of new drugs. The use of the cell proliferation kinetics and the mitotic index are recognized biomarkers in biological monitoring to evaluate cellular proliferation in population as well as to evaluate normal or tumor cells (Norppa *et al.*, 2006). Moreover, chromosomal aberrations micronucleus test have been known as sensitive methods of genetic toxicity tests of plant extracts both *in vivo* and *in vitro* (Chakrabati, 2001).

The South-West region of Saudi Arabia is rich in wild plants representing about 70% of total flora of Saudi Arabia. Apocynaceae is one of the largest plant families in the area with many species that are used to treat diseases by traditional medicine practitioners of Saudi Arabia (Rahman *et al.*, 2004).

The genus *Caralluma* belongs to the family Apocynaceae (subfamily Asclepiadoideae), which consists of about 250 genera and 2600 species. The plants belong to this genus are rich in pregnane glycosides, some of which showed antitumor activity (Deepak *et al.*, 1997). Certain species of *Caralluma* are edible and form part of the traditional medicine system for many countries (Abdel-Sattar *et al.*, 2007). These common types used in folk medicine as a remedy for treating a wide range of diseases and health conditions (Ahmad *et al.*, 1983).

In Saudi Arabia, this genus is represented by 14 species found in West and South-West regions (Chaudhary, 2001). *Caralluma* has significant anti-inflammatory activity, antinociceptive activity and antitumor activity (Deepak *et al.*, 1997; Ramesh *et al.*, 1999; Zakaria *et al.*, 2001, Rao *et al.*, 1998, Albalawi *et al.*, 2015; Priya *et al.*, 2014), cytoprotective and antiulcer activity (Al-Harbi *et al.*, 1994; Zakaria *et al.*, 2002), antioxidant, hypolipidemic (Tatiya *et al.*, 2010), antihyperglycemic (Venktesh *et al.*, 2003), antidiabetic (Wadood *et al.*, 1989), treating joint pains, paralysis, and antipyretic (Khan and Khatoon, 2008). Some species have been used as traditional and modern dietary ingredients to suppress appetite (Lawrence and Choudhary, 2004; Dutt *et al.*, 2011). To date, there are no scientific studies on genotoxic effects of *C. acutangula*. Therefore, we designed the current study to assess the impact of genetic toxicity of *C. acutangula* extract using two cytogenetics end points, chromosomal aberrations and micronucleus assays in mice bone marrow cells *in vivo*.

MATERIALS AND METHODS

Collection of plant material:

Mature *Caralluma acutangula* plants were collected from different locations from South-West region of Saudi Arabia and was identified by Dr. Yahya Masrahi, (Biological Science Department, Faculty of Science, Jazan University, Jazan, Saudi Arabia).

Extraction:

The plant was washed and dried. Then, it was grinded to fine powder, passed through a 24-mesh sieve, weighted, and stored at room temperature. The powdered sample (30 g) of *C. acutangula* was extracted with 300 ml of methanol using magnetic stirrer and stir 3 hrs at room temperature. The extract was then filtered using Whatmann No. 1 filter paper. The residue was dissolved in 300 ml methanol and stirred for 3 hrs. The solvent containing the extract was dried under reduced pressure. An aqueous extract was also prepared using 20 g powdered sample in 200 ml distilled water and stir 3 hrs at room temperature. The supernatant was boiled up to minimum volume.

Animals

Experimental study was carried out using albino mice, weighing 25-30 g. The animals were randomly assigned to different control and treatment groups (five animals in each group) that were maintained under standard conditions of humidity, temperature and light (12hr light/12hr dark), with free access to laboratory diet chow and tap water. The Institutional Animal Ethics Committee at Biological Science Department of Faculty of Science, Jazan University has approved the study.

Chromosomal preparation:

Chromosomal preparations were made according to the method described previously (Preston *et al.*, 1987). Colchicine (2.5 mg/kg body weight) was injected intraperitoneally. Animals were sacrificed by cervical dislocation 2 hr after colchicine injections. The bone marrow cells were flushed out with buffer solution (pH=7) from both femurs. The cells were centrifuged for 10 minutes at 1000 r.p.m. After centrifugation, the supernatant fluid was discarded, and the cells were resuspended in prewarmed hypotonic 0.56% KCl solution, incubated at 37°C for 25 minutes and centrifuged for 10 minutes at 1000 r.p.m. Cells were then fixed in freshly prepared Carnoy's fixative (3:1 methanol-glacial acetic acid) for 20 minutes. Fixative was changed two times. Few drops of the final suspension were dropped on clean ice-cold slides. During dropping, the slides were placed on hot plate, air-dried and stained with Giemsa stain. For each animal, at least 50 metaphases were analyzed for chromosomal aberrations using light research microscope with 1000 X magnification.

Micronucleus test (MNT):

Bone marrow smears were prepared according to the method described previously (Schmid, 1975). Bone marrow cells were flushed out in fetal calf serum, then centrifuged for 10 minutes at 1500 r.p.m., the supernatant fluid was removed and the sedimented cells were resuspended in few drops of fetal calf serum. Bone marrow cells was then smeared on a clean, dry slide. Slides were aged overnight and fixed in 70% methyl alcohol for 15 minutes. The slides were then stained for 10 minutes using Giemsa stain, rinsed in distilled water, blotted with filter paper and examined. For micronucleus assay, 2000 polychromatic erythrocytes (PCEs) were examined under 1000 X objective lens research microscope. The micronucleated PCEs were registered. A total of about 1000 erythrocytes of both types PCEs and NCEs were counted for each animal to determine the PCEs/NCEs ratio.

Statistical analysis:

Incidence of abnormal metaphases and micronucleated cells were analyzed for significance using Student's t- test.

RESULTS**Chromosomal aberrations:**

Table (I) shows the frequency of chromosomal aberrations induced in mouse bone marrow cells after single oral dose administration of *C. acutangula* extract at 125, 250 and 500 mg/kg body weight after 24 hr. The chromosomal aberration data were evaluated as the percentage of aberrant metaphase cells (including and excluding gaps). No significant increase in different types of chromosomal aberrations was detected compared with vehicle control group. Whereas, cyclophosphamide treated animals showed significant increase ($p < 0.001$) in different types of chromosomal aberrations (Table 1).

Table 1: Different types of chromosomal aberrations in mouse bone-marrow cells after 24 hr. of treatment.

Treatment	Structural chromosomal aberrations/300 cells							No. of abnormal metaphases					
								With gaps			Without gaps		
	C g	d	R T	e to e a and/or r	a f and/or b	dic	More than one type of aberrations	N o.	Means %	±SE	N o.	Means %	±SE
Vehicle control	12	4	5	1	2	7	10	41	22.77	0.601	29	16.11	0.307
Cyclophosphamide	35	7	9	8	6	26	15	106	58.89	0.988* **	71	39.4	1.222* **
<i>C. acutangula</i> extract 125 mg/kg	10	3	4	1	1	4	5	28	19.78	0.403	23	15.01	0.211
<i>C. acutangula</i> extract 250 mg/kg	11	4	3	0	2	5	6	25	17.67	0.333	20	15.11	0.201
<i>C. acutangula</i> extract 500 mg/kg	12	3	4	2	2	4	6	33	20.21	0.521	27	16.34	0.289

C g=chromatid and/or chromosomal gap d=deletion R T=Robertsonian translocation e to e a= end to end association r=ring a f=acentric fragment b=break dic=dicentric *** $p \leq 0.001$ = very highly significant

Micronucleus test

Frequency and percentage of MNPCE, and percent of PCE were shown in (Table 2). Animals treated with cyclophosphamide showed a significant increase ($p < 0.001$) in the average number and percentage of MNPCE, and percent of PCE when compared to the control group. Animals treated with different concentrations of *C. acutangula* extract showed no significant increase in the mean number or percentage of MNPCE and in the percentage of PCE when compared with control group.

Table 2: Frequency and percentage of MNPCE, and Percentage of PCE after treatment with *Caralluma acutangula* Extract

Treatment	MNPCE/2000	Percentage of MNPCE	Percentage of PCE
Vehicle control	2.11±0.29	0.12±0.01	46.31±1.9
Cyclophosphamide	13.5±1.23***	0.67±0.05***	65.99±2.03***
<i>C. acutangula</i> extract 125 mg/kg	1.9±0.34	0.09±0.01	49.34±1.3
<i>C. acutangula</i> extract 250 mg/kg	2.7±0.31	0.13±0.01	50.95±1.33
<i>C. acutangula</i> extract 500 mg/kg	2.9±0.39	0.16±0.02	51.9±0.84

***p≤0.001= very highly significant

Mitotic index

The percentage of mitotic index used to determine the rate of cell division. No significant differences in the percentages of mitotic indices were observed at the three different concentrations of *C. acutangula* extract. Cyclophosphamide treated animals showed a significant decrease (p<0.001) in percentage of mitotic index when compared with control group (Table 3).

Table 3: Effect of *C. acutangula* extract on the Mitotic index after 24 h of Treatment.

Treatment	No. of non-dividing cells	No. of dividing cells	Mitotic Index %
Vehicle control	866.30±24.33	131.70±24.33	15.24±3.15
Cyclophosphamide	976.20±6.02	23.80±6.02	2.44±0.63***
<i>C. acutangula</i> extract 125 mg/kg	887.70±8.95	112.30±8.96	12.66±1.13
<i>C. acutangula</i> extract 250 mg/kg	858.70±9.82	141.30±9.82	16.47±1.33
<i>C. acutangula</i> extract 500 mg/kg	873.2±9.30	126.8±9.35	16.57±1.23

***p≤0.001= very highly significant

DISCUSSION

In the present study, the chromosomal aberration and micronucleus assay were cooperatively used to study the genotoxic effect of *C. acutangula* extract against cyclophosphamide induced genotoxicity at a chromosomal level, micronuclei level for 24 h. It has been reported that genotoxic chemicals in general produce the highest frequency of aberrations in rodents 24 h after single exposure, which roughly coincides with the normal length (22–24 h) of the cell cycle (Adler, 1984; Hayashi *et al.*, 1984). Three genotoxicity evaluation methods cover different aspects of genotoxic events. Bone marrow chromosomal aberration is one of the most effective and sensitive short-term *in vivo* bioassay that utilizes cytological damage as an end-point in detecting and screening chemical agents that induce clastogenicity (Sanchez *et al.*, 1988).

Chromosome analysis of bone marrow cells *in vivo* is a standard method for testing for the potential mutagenic effects (Celik *et al.*, 2003). The micronucleus assay detects chromosomal damage that persists

for at least one mitotic cycle (Zhong *et al.*, 2001). Some of the DNA lesions, such as single and double strand breaks, and adduct formation, led to permanent fetal damage, and the micronucleus test is an excellent means of evaluating any permanent damage in genetic material.

Genotoxic factors may cause genetic damage in any cell of the body. In case of damage, in somatic cells, it could lead to cancer or maybe some degenerative diseases in individuals at risk. Anti-genotoxicity studies are related directly to the protective role of factors related to the genetic material. There are many reports on the investigation of anti-genotoxicity using chromosomal aberration test and micronuclei assay (Shetty *et al.*, 2010). Mutagenicity testing has been recognized as an essential part in evaluating the genotoxicity of plant extracts. This interesting idea of utilizing nuclear anomalies in bone marrow cells as a rapid method to analyze the mutagenic potential of plant extracts, provide an incentive for others who work in the field of mutation (Al-Azzawi, 2012). The rodent (mouse and rat) micronucleus assay was performed to assess the correlation between micronucleus induction and human carcinogenicity *in vivo* (Morita *et al.*, 1997).

It is acknowledged that an increase in the frequency of chromosomal aberrations in bone marrow cells is associated with an increased overall risk of cancer (Hagmar *et al.*, 1994). An increase in chromosomal aberrations may result from interactions of a wide range of chemical agents with DNA. According to Ishidate *et al.* (1988), the agents which induce an increase in the chromosomal aberrations frequency by direct or indirect mechanism may also be cytotoxic, for damage to both DNA and other cell targets (enzymes, membranes, structural proteins). Chromosomal aberrations and sister chromatid exchanges are extremely valuable and highly relevant endpoints for the detection of potential carcinogens (Swierenga *et al.*, 1991). Most of the chromosomal aberrations observed in the cells are lethal, but there are many other aberrations that are viable and cause genetic effects, either somatic or inherited (Swierenga *et al.*, 1991).

Mitotic and replication indexes are used as indicators of insufficient cell proliferation biomarkers. Rojas *et al.* (1993) suggested that cell proliferation kinetics and mitotic index measurements are useful for the prescribing of drugs with potential cytostatic activity. Some medicinal herbs show cytotoxic effect, evidenced by the decrease of the mitotic index (Gadano *et al.*, 2006).

The MNPCEs in young erythrocytes arise primarily from chromosome fragments that are not incorporated into the daughter nuclei at the time of cell division in the erythropoietic blast cells and changes in the incidence of MNPCEs are considered to reflect chromosomal damage (Salamone and Heddle, 1983).

In the micronucleus formation, when a bone marrow erythroblast develops into a PCE, the main nucleus is extruded; any micronucleus that has been formed may remain behind in the otherwise anucleated cytoplasm. Visualization of micronuclei is facilitated in these cells because they lack a main nucleus. They provide a quantifiable measure of recent DNA injury that result from acentric fragments or whole chromosomes left behind the main nucleus at telophase. An increase in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) in treated animals is an indication of induced chromosomal damage (Mavournin *et al.*, 1990; OECD 474, 1997; Gonshebbatt *et al.*, 1997; Mahata *et al.*, 2003).

To the best of our knowledge, this is the first study to investigate the effects of this plant using chromosomal aberrations test and micronucleus assay. Determination of the genotoxic/non-genotoxic effects of this plant in an *in vivo* system is important not only to prove its safety, but also to assess the potential risks when combined with pharmacologic drugs. In our previous study, we concluded that the methanolic extract of *Caralluma acutangula* possess anticancer properties *in vitro* against hepatocellular carcinoma HEPG2 cell line and breast carcinoma MCF-7 cell line (Al-Faifi *et al.*, 2016). In the current study, the *C. acutangula* extract was evaluated for genotoxic potential by means of chromosomal aberrations assay and micronucleus test in mice bone marrow cells *in vivo*. The extract did not significantly increase the number of structural chromosomal aberrations. No significant differences in the percentages of

mitotic indices were observed at the three different concentrations of *C. acutangula* extract. In micronucleus test, the extract caused no significant increase in the number of micronucleated polychromatic erythrocytes or in the mean ratio of polychromatic to total erythrocytes indicating non clastogenicity. In light of these results, the methanolic extract of *C. acutangula* does not show genotoxic effects on mice bone marrow cells *in vivo*.

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