Potential Protective Effect of *Costus speciosus* or its Nanoparticles on Streptozotocin-induced Genotoxicity and Histopathological Alterations in Rats

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Introduction

*Costus speciosus* (Family: Costaceae) is an important medicinal plant widely used in several indigenous systems of medicine for the treatment of various ailments. The rhizome of these plants are used as an alternative source for diosgenin and generally used to control diabetes. Nurseries are now promoting this exotic species as an ‘antidiabetic plant’ which lowers the blood sugar levels [1].

*C. speciosus* has an antioxidant activity which may be due to plant-derived antioxidants such as tannins, lignans, stilbenes, cou-marin, quinones, xanthones, phenolic acids, flavones, flavonols, catechins, anthocyanins and proanthocyanins that could delay or provide protection for living organisms from damage caused by uncontrolled production of reactive oxygen species and the concomitant lipid peroxidation, protein damage, and DNA strand breaking [2]. Scientific evidences suggest that antioxidants reduce risk for chronic diseases including cancer and heart disease [3].

Streptozotocin (STZ) is an antibiotic isolated from Streptomyces a chromogenes which exhibits marked antileukemic activity [4]. This compound has antibacterial, tumoricidal, carcinogenic and diabetogenic properties [5]. Chemically, STZ is an N-nitrosourea, and is related to agents such as 1,3-bis-2-chloroethyl-1-nitrosourea (BCNU), a nitrogen must-tard derivative that is commonly used in cancer therapy [6]. Because STZ is usually used to experimentally induce diabetes mellitus in laboratory animals and since it has been considered as a potential compound for the clinical treatment of malignant diseases, there is an intensive search to establish the exact mechanisms underlying cytotoxicity by STZ. It has been shown that STZ is a potent alkylating agent which directly methylates DNA [7] giving rise to chromosome and DNA damage [8]. At the chromosome level, STZ induces chromosomal aberrations (CAs) and sister-chromatid exchanges (SCEs) in mammalian and insect cells [9]. The precise mechanisms by which STZ induces CAs and SCEs remain unknown. Previous reports provided evidence that free radicals may be involved in the diabetogenic action of the drug [10]. Bolzan et al., [9] demonstrated that STZ-induced CAs in CHO and mosquito cells can be prevented to a great extent by the incorporation of antioxidant compounds into the cells. This finding suggests that free radicals are involved in the clastogenesis by STZ.

The genotoxic effects of STZ indicates that this compound is highly mutagenic, causing DNA and chromosome damage by mechanisms which involve free radicals generated during STZ metabolism. DNA lesions produced by STZ include monoadducts, double and single-strand breaks, and alkali-labile sites. Severe DNA damage by STZ results in cell death by apoptosis or necrosis. At the chromosome level, STZ induces micronuclei, CAs and SCEs. Despite the involvement of free radicals in the production of DNA and CAs by STZ, recent evidence suggests that these active oxygen species are not involved in the induction of SCEs by this compound. Very likely, STZ-induced SCEs from the alkylating action of the antibiotic on the DNA molecule. Furthermore, although both O6-MeGand N7-MeG adducts can be converted to CAs and SCEs, since more than 70% of DNA methylation by STZ occurs at the N7 position of guanine, N7-MeGseems to be the predominant clastogenic and recombinogenic lesion for this compound [11]. Histopathological studies of Dhavanath [12] on pancreas of STZ-treated diabetic rats showed generation of pancreatic islets of rats, as confirmed by...
immunohistochemical studies. However, the treatment with Swertiamarin (a flavone like costus) which considered as antioxidant, antimutagenic, hepatoprotective and anticancer and contains protective agents like saponins, catechins, sterols, steroids, triterpenoids, alkaloids, and volatile oil showed regeneration of these islets confirmed the cytoprotective nature of these compounds. In addition, vacuolar degenerative changes in hepatic cells with damaged central vein and portal triad were seen in STZ-induced diabetic rats. These potent damages were completely reversed in all swertiamarin-treated groups [12]. Histopathological studies were also carried out by Anitha [13] to assess the effect of chrysin as a flavon on liver, kidney and pancreatic cells against STZ-induced damage in rats and concluded that chrysin a flavon can act as a potent antioxidant and anti-inflammatory agent in STZ-induced type II diabetic rats. As well Mir et al. [14] reported histomorphological alterations of pancreas, kidneys, liver, heart, lungs and brain in STZ treated rabbits. Therefore, the present study was conducted to investigate the potential protective effect of the traditional plant Costus speciosus (Koen.) or nanocostus that is used frequently for the treatment of various disorders specially diabetes against genotoxicity and histopathological alterations induced by STZ in male rats.

Materials and Methods

Animals

Male albino Wistar rats were purchased from the laboratory of animal house, National Research Center, Dokki, Cairo, Egypt and were kept 1 week for aclimatisation and supplied with feed and water ad libitum. Animals were divided into 8 groups (10 animals/group) and treated according to Gireesh [15] as follows: 1) Streptozotocin (ip single dose of 80 mg/kg STZ, 3 d after the blood sugar was 200-250 mg/dl); 2) STZ + insulin (1 U for 14 days starting from d3 after STZ injection); 3) STZ + 50 mg/kg costus (oral administration); 4) STZ + 100 mg/kg costus; 5) STZ+150 mg/kg costus; 6) STZ + 50 mg/kg nanocostus (oral injection); 7) STZ + 100 mg/kg nanocostus; 8) STZ + 150 mg/kg nanocostus. Beside the control group (received only the vehicle of 0.1 M citrate buffer; 1 ml/kg bw).

Chemicals and Diabetes induction

Streptozotocin (STZ) was purchased from Sigma–Aldrich (Sigma-Aldrich 3505 Spruce St., St. Louis, MO 63103, USA). Diabetes mellitus was induced by single intra-peritoneal injection of freshly prepared STZ (50 mg/kg bw) [16], dissolved in 0.1 M citrate buffer (pH 4.5) in a volume of 1 ml/kg bw. Diabetes was developed and stabilized in these STZ-treated rats over a period of 3 - 4 days. The control animals were administered with citrate buffer (pH 4.5). After 3 days, the blood was collected by sinocular puncture and the plasma glucose level of each rat was determined. Rats with a fasting plasma glucose range of 280-350 mg/dl were considered diabetic and included in the study [17].

Costus speciosus preparation

Costus speciosus leaves were grinded and boiled in water for 30 min, filtered and evaporated by evaporator. The extract was dried by freeze dry as water extract of PE (PEW). The percentage of yield obtained as 43.4%. The samples have been preserved in the refrigerator (~20 °C). Preparation of the extract was accomplished according to Tasanarong [18]. Formation of Costus speciosus nanoparticles was achieved using solvent displacement technique [19].

Comet assay

Comet assay was performed using blood samples under alkaline conditions [20] with slight modification. Slides were scored using Comet Score Software (Tritek Corp., USA) and 100 cells were analyzed per sample. The parameters used to assess DNA damage were tail length or class of comet (migration of DNA from nucleus) and DNA damage frequency. The control and treated slides were randomized and were not run separately or at different times to avoid variability.

Micronucleus assay

Micronucleus test was performed using the method of Igarashi [21], with slight modification. A small drop of blood was placed at one end of clean, grease free microscopic slide. The drop was carefully spread into a single cell layered film without damaging the cell morphology using a polished cover glass held at an angle of 45°. The slides were air dried for 12 h and subsequently stained for 1 - 2 min in concentrated May–Grunwald stain (0. 25% in methanol) followed by 10% Giemsa stain solution for 10 min [22]. The slides were then rinsed twice with distilled water, dried and rinsed with methanol. The slides were placed in xylene for clearing, mounted in DPX and analyzed for the presence of micronuclei (a total of 1000 cells were scored for each animal).

Chromosomal aberrations analysis

The rats were sacrificed 24 h after administration of the last treatment for chromosome aberration analysis. Cytogenetic analysis was performed on tibia bone marrow cells according to the recommendations of Adle [23], with slight modifications. Experimental animals were injected (i.p.) with colchichines (4 mg/kg) 1.5 h before sacrifice. Both tibia were dissected out and cleaned of any adhering muscle. Bone marrow cells were collected from both tibias by flushing in KCl (0.075 M, at 37 °C, 5 mL) and incubated at 37 °C for 30 min. Material was centrifuged at 2000 rpm for 10 min, fixed in methanol: acetic acid (Carnoy’s fixative, 3:1 v/v). Cytogenetic analysis and fixation (in the cold) were repeated five times at least at intervals of 20 min. The material was resuspended in a little volume of fixative, dropped onto chilled slides, flame-dried and stained in 5% Sorenson buffered Giemsa (pH: 6.8). At least 75 good metaphases containing 42 chromosomes were examined per animal to score different types of aberrations.

For spermatocyte cells, chromosomal preparations were made according to the air- drying method Evans [24]. Rats were injected (i.p.) with colchichines (0.1%) 2 h before killing by cervical dislocation. The testes were transferred to 2.5 ml of a 2.2% citrate solution in Petri dishes and the tunica removed. The contents of the tubules were gently teased out with curved forceps. The cell suspension produced was aspirated well and centrifuged at 1000 rpm for 10 min the supernatant was discarded, and the pellet was resuspended in 2 ml of hypotonic solution (1% sodium citrate) at 37°C. After 12 min, the suspension was centrifuged for 10 min at 1000 rpm. Then the supernatant was removed. The cells were fixed 3 times with cold fixative solution (3:1 of methanol and glacial acetic acid). Slides were stained with Giemsa in phosphate buffer (pH 6.8) for 8 min. Fifty primary spermatocytes/rat at diakinesis-metaphase I were scored. Abnormalities recorded included univalents (x–y univalent, autosomal univalent), chains, rings, N ± 1 and polyploidy.
Histopathological analysis

Specimens (liver and pancreas) of all animals were dissected immediately after death, washed thoroughly with formal saline and then fixed in 10% neutral-buffered formal saline for 72 hours at least. All the specimens were washed in tap water for half an hour, dehydrated in ascending grades of alcohol (70%-90%-95%-absolute), cleared in xylene and then embedded in paraffin wax. Serial sections of 6 µm thick were cut and stained with Haematoxylin and eosin for histopathological investigation [25].

Statistical analysis

Data were compared by one-way analysis of variance (ANOVA). Statistical analysis was performed using the SPSS for Windows 9.05 package program. Multiple comparisons were carried out by least significant difference (LSD) test. P < 0.05 was considered as the level of significance.

Results and Discussion

Cyto genetic analysis

The potential antigenotoxic effect of the aqueous extract of C. speciosus on STZ treated male rats were evaluated using comet assay, micronucleus test and chromosomal aberration analysis. The results in the present investigation exhibited the ability of C. speciosus as antigenotoxic potential against STZ induced DNA damage, micronucleus (Mn) and chromosomal aberrations (CA) in male rats.

Comet assay

In the present study DNA assay damage was evaluated by comet assay. Administration of STZ resulted in DNA damage as is evident in Table 1. It is evident that exposure to STZ resulted in DNA damage (24.3%) as compared to control group (6.7%). However, costus or nanocostus treatment in combination with STZ reduced significantly DNA damage frequency especially with the dose of 150 mg nanocostus (11.7%). These results suggest that costus or nanocostus has a protective effect on STZ- induced DNA and prevent genotoxicity induced by STZ. This coincides with Murata et al. [26] who suggested that STZ induces DNA damage by methylation of guanines via methyl cations. As well with Eliza et al. [27] who found that the treatment with costus extract restored the pancreatic DNA level to near normal.

Micronucleus assay

The results of the micronucleus frequency (Table 2) revealed a significant increase in micronucleated polychromatic erythrocytes frequency in all treated groups compared to control, however the treatment with costus lowered the incidence of MN to be closed to the control group especially with 50 mg/kg bw nanocostus (8.60 ± 0.89 for STZ + 50 mg/kg bw nanocostus vs. 5.60 ± 1.14 for the control group). That in accordance with Bolzán et al. [11] who reported that the genotoxic effects of STZ indicates that this compound is highly mutagenic and induce micronuclei by mechanisms which involve free radicals generated during STZ metabolization.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of animals</th>
<th>No. of cells Analyzed (*)</th>
<th>Total comets</th>
<th>Class# of comet (DNA damaged cells (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>300 (7+6+7)</td>
<td>20</td>
<td>280</td>
</tr>
<tr>
<td>STZ</td>
<td>3</td>
<td>300 (25+24+24)</td>
<td>73</td>
<td>227</td>
</tr>
<tr>
<td>STZ+Insulin</td>
<td>3</td>
<td>300 (14+13+15)</td>
<td>42</td>
<td>258</td>
</tr>
<tr>
<td>STZ+50 mg costus</td>
<td>3</td>
<td>300 (19+20+21)</td>
<td>60</td>
<td>240</td>
</tr>
<tr>
<td>STZ+100 mg costus</td>
<td>3</td>
<td>300 (18+17+18)</td>
<td>53</td>
<td>247</td>
</tr>
<tr>
<td>STZ+ 150 costus</td>
<td>3</td>
<td>300 (14+16+15)</td>
<td>45</td>
<td>255</td>
</tr>
<tr>
<td>STZ+50 mg nanocostus</td>
<td>3</td>
<td>300 (15+16+16)</td>
<td>47</td>
<td>253</td>
</tr>
<tr>
<td>STZ+100 mg nanocostus</td>
<td>3</td>
<td>300 (11+13+12)</td>
<td>36</td>
<td>264</td>
</tr>
<tr>
<td>STZ+150 mg nanocostus</td>
<td>3</td>
<td>300 (12+11+12)</td>
<td>35</td>
<td>265</td>
</tr>
</tbody>
</table>

Table 1: Visual score of DNA damage in blood samples of male rats exposed to streptozotocin (STZ) and/or costus or nanocostus extracts using comet assay. ¥: Class 0 = no tail; 1 = tail length < diameter of nucleus; 2 = tail length between 1X and 2X the diameter of nucleus; and 3 = tail length > 2X the diameter of nucleus. (*): No of cells analyzed were 100 per animal.

However, the treatment with C. speciosus that has an antioxidant activity which may be due toplant-derived antioxidants such as tannins, lignans, stilbenes, cou-marins, quinones, xanthones, phenolic acids, flavones, flavonoids, catechins, anthocyanins and proanthocyanins that could delay or provide protection for living organisms from damage caused by uncontrolled production of reactive oxygen species and the concomitant lipid peroxidation [2,3].

Chromosomal aberrations analysis

The metaphase analysis of bone marrow cells (Table 3) shows that the more frequent types of chromosomal aberrations were chromatid gaps and break, chromosome gap, fragment, deletions and endomiosis. Also, the numerical variations (N ± 1 and polyploidy) were observed to be more frequent in all treated groups except for STZ +50 mg/kg bw nanocostus treated group compared to control.
exchanges (SCEs) in mammalian and insect cells [9]. Previous report
polyploidy) compared to the control group. At the chromosome level,
to control (P<0.05). However the treatment with costus and/or
costus and/or costus or nanocostus. Small different superscript letters
Table 2: Micronucleated polychromatic erythrocytes of male rat treated with STZ and/or costus or nanocostus. Small different superscript letters are differing significantly.

The frequency of chromosomal aberrations in spermatocyte cells (Table 4) significantly increased with STZ treatment when compared to control (P<0.05). However the treatment with costus and/or nanocostus in combination with STZ lowered the percentage of all types of structural (x-y univalents, chain, ring and autosomal univalents) aberrations and numerical variations (N ± 1 and polyploidy) compared to the control group. At the chromosome level, STZ induces chromosomal aberrations (CAs) and sister-chromatid exchanges (SCEs) in mammalian and insect cells [9]. Previous report
Ohkuwa et al. [10] provided evidence that free radicals may be involved in the diabetogenic action of the drug. In addition, studies of Bolzan et al. [9] demonstrated that STZ-induced CAs in CHO and mosquito cells can be prevented to a great extent by the incorporation of antioxidant compounds into the cells. This finding suggests that free radicals are involved in the clastogenesis by STZ. However, costus treatment provide protection for living organisms from damage caused by uncontrolled production of reactive oxygen species [2, 3].

Table 2: Micronucleated polychromatic erythrocytes of male rat treated with STZ and/or costus or nanocostus. Small different superscript letters are differing significantly.

Table 3: Mean percentages ± SE of chromosomal aberrations in rat bone marrow cells after treatment with STZ and/or costus or nanocostus. Small different superscript letters are differing significantly.
The protective effect of *C. speciosus* is due to its antioxidant action, trapping of free radicals, formation of complex with mutagens [28]. The mode of action of anti-mutagenesis may act as modulation of mutagen metabolism by absorbing the xenobiotics, or inhibition of SOS (superoxide oxygen species) functions or by altering the activation and detoxification of toxic agents as suggested by similar results obtained by [29]. Also, the stabilization of the formed phenoxy free radicals is responsible for its free radical scavenging activity and chemopreventive effect mutagens [30]. The modulatory role of *C. speciosus* in inhibiting mutagenicity and/or cytotoxicity need more studies to understand the mechanism of antigenotoxic action.

**Histopathology of liver and pancreas**

From the pathological point of view, the lesions due to STZ toxicity were commonly noticed in liver and pancreas. (Fig. 1, A) showed the normal structure of liver tissue in control rat. Treatment of diabetic rats with coctus revealed dose dependent amelioration of the damaging effect of STZ inducing diabetes on liver tissue (Figure 1 B,C and D). The same results were obtained in case of diabetic rats treated with nanocostus (Figure 2 A, B and C). Results obtained from diabetic rats treated with insulin were better than those obtained from samples treated with 50mg/kg bw nanocostus but less than those obtained from samples treated with 100 and 150 mg/kg bw nanocostus (Figure 2 D).

Examination of pancreatic samples showed the normal structure of this tissue as in (Figure 3A). The coctus treatment revealed amelioration of the damaging effects of STZ induced diabetes on pancreatic tissue in a dose dependent manner (Figure 3 B,C and D). While, using nano technique in the treatment of diabetes revealed that the best amelioration were obtained with 50mg/kg bw nanocostus, where restoration of the normal size and shape of island of Langerhans was achieved (Fig. 4 A), but less restoration were obtained in case of 100 and 150 mg/kg bw nanocostus (Figure 4 B,C). While, treating diabetic rats with insulin (Figure 4D) revealed that insulin gave results less than those given with 50 mg/kg bw nanocostus but better than those given with 100 and 150 mg/kg bw nanocostus.

These findings coincide with those of [12] who found that pancreas of STZ-treated diabetic rats showed generation of pancreatic islets. While treatment with Swertiamarin (a flavones like costus) which considered antioxidant and anticancer and rebuild liver cells and contains protective agents like saponins and catechins showed regeneration of these islets. As well, vacuolar degenerative changes in hepatic cells with damaged central vein and portal triad were seen in STZ-induced diabetic rats were completely reversed in all swertiamarin-treated groups [12]. The same results were achieved by [13] indicating the antioxidant and anti-inflammatory effects of these compounds.

Table 4: Mean percentages of chromosomal aberrations in rat spermatocytes after treatment with STZ and/or costus or nanocostus. Small different superscript letters are differing significantly.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chromosomal Aberrations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STZ + 50 mg nanocostus</td>
<td>250 (4, 1.6b)</td>
</tr>
<tr>
<td>STZ + 100 mg nanocostus</td>
<td>250 (8, 3.2C)</td>
</tr>
<tr>
<td>STZ + 150 mg nanocostus</td>
<td>250 (13, 5.2d)</td>
</tr>
</tbody>
</table>

Figure 1: A photomicrograph of sections of liver tissue (A) for control rat shows the normal structure of liver tissue. (B) For a diabetic rat received 50mg Coctus shows congestion of main blood vessels, vacuolar degeneration of hepatocytes (arrowhead) and dilatation of blood sinusoids (arrow). (C) For a diabetic rat received 100mg Coctus shows thickening of blood vessels' wall (arrow), mild vacuolar degeneration of hepatocytes (arrowhead) and cellular infiltration (in the lower right corner of figure). (D) For a diabetic rat received 150mg Coctus shows restoration of normal structure of liver tissue but with mild dilatation of blood sinusoids. (Hx. & E. X 200).

In conclusion, it can be stated that the treatment with STZ induced genotoxicity and histopathological alterations in rats and that appeared in the form of significant increase in the frequency of DNA damage, micronuclei, chromosomal aberrations and histopathological changes. However, the treatment with costus or nanocostus reduced these toxicities especially with nanocostus, indicating the protective nature of these compounds.

Figure 2: A photomicrograph of sections of liver tissue (A) for a diabetic rat received (Nano 50) shows distortion of normal architecture of liver tissue, congestion of main blood vessels, dilatation of blood sinusoids and apoptotic and vacuolar degenerated cells. (B) For a diabetic rat received (Nano 100) shows restoration of normal architecture of tissue but with vacuolar degeneration of some cells (arrow) and acidified cells (arrowhead). (C) For a diabetic rat received (Nano 150) shows dilatation of blood sinusoids (arrow) with increased number of Kupffer cells (arrowhead). Minimal degree of vacuolar degeneration is observed. (D) For a diabetic rat received insulin shows marked dilatation and congestion of blood sinusoids with no signs of vacuolar degeneration. (Hx. & E. X 200).

Figure 3: A photomicrograph of sections of pancreatic tissue (A) for a control rat shows islands of Langerhan (IL), serous acini (arrowhead) and ducts (arrow). (B) For a diabetic rat received 50mg Coctus shows atrophy of islands of Langerhan (arrow) with vacuolar degeneration of many of its cells. (C) For a diabetic rat received 100mg Coctus shows mild amelioration of islands of Langerhan. (D) For a diabetic rat received 150mg Coctus shows restoration of normal size and shape of island of Langerhan with no evidence of vacuolar degeneration. (Hx. & E. X 200).

Figure 4: A photomicrograph of sections of pancreatic tissue (A) for a diabetic rat received (Nano 50) shows restoration of the normal size and shape of island of Langerhan but with mild vacuolar degeneration of some cells. (B) For a diabetic rat received (Nano 100) shows atrophy of island of Langerhan. (C) For a diabetic rat received (Nano 150) shows normal size of island of Langerhan with vacuolar degeneration of some cells (arrowhead) and at the periphery of the island. (D) For a diabetic rat received insulin shows atrophy of the island of Langerhan but with no signs of vacuolar degeneration. (Hx. & E. X 200).

References
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