TINOSPORA CRISPA IS AMELIORATING CISPLATIN-INDUCED CYTOTOXICITY AND GENOTOXICITY IN BREAST EPITHELIAL CELLS

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**Abstract**

Effective anticancer therapy with cytotoxic drugs such as Cisplatin is limited by severe side effects due to bystander damage to the normal cells. Thus, in this study, we aimed to investigate the possible protective effect of *T. crispa* on Cisplatin-induced cytotoxicity and genotoxicity in non-cancerous breast epithelial cells, MCF-10A. Our results showed that Cisplatin increased the cell death in MCF-10A cells in a dose dependent manner, with the IC\(_{50}\) of 18 ± 2 \(\mu\)g/ml. Interestingly *T. crispa* showed very little toxicity to the MCF-10A cells, with the IC\(_{50}\) of 248 ± 7 \(\mu\)g/ml. Furthermore, when the MCF-10A cells were treated with Cisplatin in the presence of *T. crispa*, the cell cytotoxicity was significantly decreased \((p<0.05)\) as compared to Cisplatin alone. The cell death mechanism induced by both compounds was investigated using cellular DNA fragmentation ELISA method. *T. crispa* decreased the apoptotic effect induced by Cisplatin in MCF-10A cells \((p<0.001)\) as compared to Cisplatin alone. Similarly, *T. crispa* decreased the genotoxic effect of Cisplatin in MCF-10A \((p<0.001)\) as compared to Cisplatin alone. These findings were reconfirmed by QPCR analysis. QPCR analysis showed that the mRNA expression levels of apoptosis related genes (caspase-3 and caspase-9) induced by Cisplatin were significantly decreased in the presence of *T. crispa* as compared to Cisplatin alone \((p<0.001)\). The mRNA level of NF-\(\kappa\)B (p50/p65) was also down-regulated indicating less susceptibility to apoptosis. In conclusion, our study showed that *T. crispa* reduced the cytotoxic, genotoxic and apoptotic effect of Cisplatin in non-cancerous breast epithelial cells.

**Keywords**

*T. crispa*, Cisplatin, Cytotoxicity, Genotoxicity, Apoptosis, MCF-10A

1.0 Introduction

Cisplatin \([\text{cis-dichlorodiammine-platinum (II), CP}]\) is an effective chemotherapy drug that has been used for the treatment of various cancers including sarcomas, small cell lung cancer, ovarian cancer, breast cancer, lymphomas and germ cell tumors. Cisplatin forms a platinum complex inside the cell by cross-linking with DNA. This will cause damage to the DNA and thus activate the DNA repair mechanism. Once the mechanism is activated, the cells will undergo apoptosis \((1-3)\). Like most chemotherapeutic drugs Cisplatin has been reported to induce significant side effects such as nephrotoxicity, neurotoxicity, hepatotoxicity, ototoxicity, alopecia, electrolyte disturbance, nausea and vomiting \((4-5)\). The putative main mechanism involved in cisplatin-induced normal tissue damage is due to the production of free radicals during its intracellular metabolism. Free radicals cause diverse oxidative damage to critical cellular components and membranes in normal tissues \((6-7)\). Thus, considerable efforts have been made to investigate the possible use of antioxidants to reduce the side effects of chemotherapeutic drug
administration in healthy cells without compromising the efficacy in cancer cells. Strategies to optimize the existing chemotherapy treatments have the potential to consolidate chemo-sensitivity and thus decreasing their side effects and increase the compliance of patients to use these drugs. Recent studies suggested that combined chemotherapy using Cisplatin and plant extracts can reduce the side effects and therefore improve the anti-proliferative activity in several types of cancers (8-10).

*T. crispa* is a well-known traditional plant used in several Asian countries and it contains important nutrients: minerals and flavonoids that are valuable for health. *T. crispa* is locally known as Batawali in Malaysia, Andawali in Indonesia, Makhamukay in Philippines and Boraphet in Thailand (11-13).

Various studies have reputed *T. crispa* efficacies as anti-proliferative agent against several types of cancers (14-17).

The molecular basis of normal tissue injury is not well understood. It is considered that several factors can play a role. Among these is; the transcriptional nuclear factor-kappa B (NF-xB) that is involved in cellular proliferation, regulation of inflammatory and apoptotic genes.

In the present study, we aimed to investigate the possible protective mechanism of *T. crispa* in cisplatin-induced cytotoxicity, genotoxicity and inflammatory properties in non-cancerous breast epithelial cells, MCF-10A.

2.0 Materials and Methods

2.1 Plant material

The raw plant materials of *T. crispa* were collected from a village in Chenor, Pahang. The stems from the plant were cut into small pieces (2 - 2.5 cm in length), dried and grounded into powder. The powder was soaked in methanol overnight at room temperature and the methanol extract was then filtered and concentrated using a rotary evaporator followed by freeze-drying. Finally, the dried extract was stored at -80°C (15). To prepare the stock solution, the dried extract was reconstituted with methanol at a concentration of 10 mg/mL and then serially diluted in growth medium to obtain the concentrations used in this study.

<table>
<thead>
<tr>
<th>Grade of genotoxicity</th>
<th>DNA damage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0 (no damage)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Grade 1 (low level damage)</td>
<td>5-20</td>
</tr>
<tr>
<td>Grade 2 (medium level damage)</td>
<td>21-40</td>
</tr>
<tr>
<td>Grade 3 (high level damage)</td>
<td>41-95</td>
</tr>
<tr>
<td>Grade 4 (total damage)</td>
<td>&gt;95</td>
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</table>

2.2 Chemicals and reagents

Cisplatin, dimethyl sulfoxide (DMSO), Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), horse serum, accutase, penicillin-streptomycin, hydrocortisone, insulin, epidermal growth factor and MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from (Invitrogen, USA). Methanol of the highest pure grade was obtained from Merck, (Germany).

2.3 Cell culture

The MCF-10A cell line was obtained from the American Type Culture Collection (Manassas, USA). MCF-10A was grown in DMEM/F12 media supplemented with 5% horse serum, 100 μg/mL hydrocortisone, 10 μg/mL insulin, 20 ng/mL epidermal growth factor and 1% penicillin-streptomycin at 37°C with 5% CO2 and 95% humidity till the culture was 70-80% confluent.

2.4 MTT assay

The effect of *T. crispa* and Cisplatin on cell cytotoxicity of non-cancerous breast epithelial cells, MCF-10A was investigated using MTT assay. 2x10^3 cells were cultured in each well of a 96 well plate in 100 μL complete culture media and were incubated overnight to allow cell attachment. Cells were treated separately with a different range of *T. crispa* and cisplatin concentrations (0 to 400 μg/mL). Cells without treatment and cells treated with Cisplatin were used as negative and positive controls respectively. The culture was incubated at 37°C in 5% CO2 for 72 hours. 10 μL of MTT-PBS (5 mg/mL) were added into each well and further incubated for 4 hours at 37°C in 5% CO2 and 95% humidity. The medium was then removed and replaced with 100 μL of DMSO to solubilize the MTT formazan product. The solution was mixed on a plate shaker for 15 minutes before measuring the absorbance at 570 nm wavelength using a microplate reader (Biomek® FX, Beckman Coulter, USA).

2.4 Cellular DNA fragmentation ELISA detection

The effect of *T. crispa* and Cisplatin on cell apoptosis was investigated using DNA Fragmentation ELISA kit (Roche Molecular Biochemicals, Germany). Confluent MCF-10A cells were adjusted to 4x10^5 cells/mL. Labelling solution (BrdU) was added to a final concentration of 10 μM followed by incubation for 18 hours at 37°C in 5% CO2. Accutase was added to detach the cells, and then cells were centrifuged for 10 minutes at 250xg and re-suspended in BrdU free culture medium. The cells were adjusted to 1x10^5 cells/mL and 100 μl of this cell suspension was pipetted per well (96-well, round-bottom microtiter plate). Cells were allowed to attach overnight, growth medium was discarded and 60 μg/ml of...
Table 2 QPCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tr>
<td>Caspase 3</td>
<td>F: 5′-GAGTGCTCGACAGCTATACCT3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CCTCACGCCTGGGATT3′</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>F: 5′-GATCGCGCTTCTTCCTTG3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTCTACACTTCAAGACAC3′</td>
</tr>
<tr>
<td>NF-κB (p50)</td>
<td>F: 5′-AACCTGACGAAGCTTCCAC3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-ACACAGGCTAGGATT3′</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5′-CCCAGCTTGCTTGAAGG3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTCTGGCTTTTGGCTTG3′</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>F: 5′-GGGCCCACAGGAGACAG3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5′-AGAGGCTCTGACAGGACG3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-TGCAACAACACTGGT3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5′-CCACGGGAGTACCTTCG3′</td>
</tr>
</tbody>
</table>

T. crispa, 18 µg/ml of Cisplatin or combination of both were added along with 100 µl/well of growth medium and the cells were further incubated for 72 hours at 37°C in 5% CO2 and 95% humidity. After that, the plate was centrifuged at 250xg for 10 minutes, media was removed and 200µL/well of the lyses solution was added to lyse the cells. After an incubation time of 30 min at room temperature the plate was centrifuged again for 10 min at 250xg. 100 µL of the centrifugation supernatant were used for the ELISA analysis. The supernatant was transferred to an anti-DNA–pre-coated microtiter plate and incubated for 90 min at room temperature. After washing the wells, the samples were denatured and fixed by microwave irradiation for 5 minutes. After the microtiter plate was cooled for 10 minutes at -20°C, conjugate solution was added, and the plate was incubated for 90 minutes at room temperature. Cells were washed again, substrate solution was added, and the plate was incubated for 30 minutes at room temperature. Absorbance was measured at 370nm. The increase in apoptotic cells is associated with an increase in absorbance. A concentration of 1x10⁶ apoptotic cells per well reflects 1.5 absorbance (O.D. reading).

2.6 Analysis of DNA Damage (Comet Assay)

DNA damage was estimated using single cell gel electrophoresis (SCGE or comet assay) (Singh et al., 1988). Fully frosted slides were covered with 0.6% of normal melting agarose (NMA) as the first layer, a mixture of cell suspension as the second layer, and finally with 0.6% of low melting agarose (LMA) as the third layer. After solidification at 4°C, all slides were immersed in the lyses buffer containing 2.5 M NaCl, 100 mM Na₂EDTA, 10 mMTris and 1% Triton-X (pH 10) at 4°C for 1 hour. The embedded nuclei were then incubated with or without the test agents (T. crispa 60 µg/ml and Cisplatin 18 µg/ml) for 4 hours at 37°C. This treatment step allows the test agent to come into direct contact with the DNA of each cell. The slides were then removed and placed in a horizontal electrophoresis tank. The tank was filled with freshly prepared electrophoresis solution consisting of 300 mM NaOH and 1 mM Na₂EDTA (pH 13). The slides were left in the solution for 20 minutes to allow DNA unwinding and expression of alkali labile damage. Electrophoresis was then conducted at 4°C for 20 minutes using 25 V and 0.3 A. After electrophoresis, the slides were neutralized in neutralization buffer, stained with SYBR® Green I nucleic acid gel stain and examined using a fluorescence microscope. The degree of DNA damage was graded into 5 categories according to the amounts of DNA damage (Table 1).

2.7 Quantitative (Real-time) polymerase chain reaction (QPCR)

MCF-10A cells were treated with 18 µg/ml of Cisplatin and/or 60 µg/ml of T. crispa for 72 hours. Total RNA was extracted from treated MCF-10A non-cancerous breast epithelial cells using RNeasy Mini kit (Qiagen, Germany). The cells were then lysed and homogenized using QIA shredder column (Qiagen, Germany). Total RNA was isolated according to manufacturer’s protocol while the concentration and purity of eluted RNA was determined using Nano drop (Nano Drop ND-1000 spectrophotometer, USA).

cDNA was produced using 1µg of total RNA in a 20µL reaction with Sensiscript RT and Oligo-dT primers. Expression of caspase-3, caspase-9, NF-κB, TNF-α and ICAM–1 were analyzed using real time PCR. A cocktail of 1x SYBR Green Supermix, 25µM forward and reverse primers, and 2µLcDNA template in a 25 µL volume was amplified using iQ™ 5 Real Time PCR detection system (Bio Rad, USA). Normalization of gene expression was done with β-
actin and GAPDH as housekeeping (reference) genes and iQ™ 5 Real Time PCR software (Bio Rad, USA) for all samples. All the QPCR primer sequences (Table 2) were purchased from Sigma (St. Louis, USA).

2.8 Statistical analysis

All data are expressed as means ± S.D from at least 3 replicates. The Statistical significance was assessed by two-tailed Student t tests for single comparisons or One-Way ANOVA followed by Bonferroni post hoc test for multiple comparisons using SPSS 16.0 for Windows (SPSS Inc., Chicago, USA). Differences at $p<0.05$ were considered statistically significant.

3.0 Results

3.1 *T. crispa* protective effect against Cisplatin-induced cytotoxicity in MCF-10A cells

To examine whether *T. crispa* has a cytotoxic effect against human MCF-10A breast epithelial cells, we performed MTT assay. MCF-10A cells were treated with *T. crispa* or cisplatin at concentrations (0, 10, 50, 100, 200 and 400 µg/ml) for 72 hours and the percentage cell death was evaluated. As showing in figure 1, *T. crispa* showed significantly less cytotoxicity against MCF-10A cells compared to cisplatin (*$p<0.05$ and **$p<0.01$). The data are presented as mean ± SD (n=6).

3.2 *T. crispa* decreased the apoptotic activity in Cisplatin-treated MCF-10A cells

Next, we investigated whether *T. crispa* protects against Cisplatin induced apoptosis in MCF-10A cells using cellular DNA fragmentation ELISA. Cisplatin is known to induce apoptotic cell death in cancer cells (1-3, 9-10, 19). One characteristic of apoptosis is the selective fragmentation and packaging of cellular DNA. The results showed a significant decrease (**$p<0.001$) in apoptotic activity when the combination of *T. crispa* with Cisplatin was used compared to Cisplatin alone indicating a protective effect for *T. crispa* against Cisplatin induced-apoptosis in MCF-10A cells.
These results are shown in figure 3.

3.3 The effect of *T. crispa* on Cisplatin-induced genotoxicity in MCF-10A cells

The effect of *T. crispa* on Cisplatin-induced genotoxicity is presented in figure 4. The data showed that MCF-10A cells treated with 18 µg/ml Cisplatin resulted in serious DNA damage. The DNA damage was mainly grade 3 and grade 4. In contrast, grade 3 and grade 4 DNA damage was significantly reduced in cells when 60 µg/ml of *T. crispa* extract were added (*p*<0.05). The results also indicated that treatment with different concentrations of *T. crispa* alone did not cause significant DNA damage in MCF-10A cells (data not shown).

3.4 The effect of *T. crispa* on the expression levels of apoptosis related genes induced by Cisplatin in MCF-10A cells

To investigate the molecular mechanism of Cisplatin-induced apoptosis in MCF-10A cells, the expression levels of caspase-3 and caspase-9 were examined. Our results showed that Cisplatin significantly up-regulated the gene expression of both caspase-3 and caspase-9. Additionally, our results showed that treatment with Cisplatin in the presence of *T. crispa* down-regulated the gene expression of both caspase-3 and caspase-9 in MCF10A as compared to Cisplatin alone (figure 5). This may indicate a possible protective effect of *T. crispa* against Cisplatin-induced apoptosis in normal cells.

3.5 The effect of *T. crispa* on the expression levels of NF-κB (p50/p65) genes induced by Cisplatin in MCF-10A cells.

The results demonstrated that Cisplatin significantly up-regulated the gene expression of NF-κB subunits [NF-κBIA (p50) and NF-κB (p65) RelA]. Aberrant regulation of NF-κB by several chemotherapeutic drugs including Cisplatin has been linked to an inflammatory response and cell death in normal tissue (20). More interestingly, the results also showed that addition of *T. crispa* to Cisplatin treatment significantly down-regulated NF-κB (p50/p65) as shown in figure 6.

3.6 The effect of *T. crispa* on the expression levels of inflammation related genes induced by Cisplatin in MCF-10A cells.

The results showed that Cisplatin significantly up-regulated the gene expression of inflammation related genes like Tumor Necrosis Factor-α (TNF-α) and Intercellular Adhesion Molecule-1 (ICAM-1). The combination of *T. crispa* and Cisplatin significantly down-regulated the gene expression of the inflammatory genes TNF-α and ICAM-1 indicating a
protective effect against Cisplatin induced inflammation as shown in figure 7.

**4.0 Discussion**

Chemotherapy is one of the most valuable methods for the treatment of cancer, but the cytotoxic activity of chemotherapeutic drugs in normal cells can induce serious side effects such as immuno-suppression, nephrotoxicity, neurotoxicity, hepatotoxicity, nausea, vomiting, hair loss, anemia and digestive disorders (2-3, 9-10). Thus, the damage to normal tissue by anti-tumor drugs can be a major limitation for patient’s tolerance to chemotherapy. Several groups have worked on chemo-protective agents in an attempt to find a preferential protection of normal tissues during cancer treatment (21-23). In the present study, we observed that treatment with Cisplatin in combination with *T. crispa* extract significantly reduced the toxic effects caused by Cisplatin in MCF-10A cells as a model for non-cancerous breast epithelial cells.

Recent studies have shown the anti-cancer potency of natural products to reduce the side effects and to improve the therapeutic effect of chemotherapeutic agents(24-26). Most of these studies focused on the synergistic effect of combined therapy in cancer cells by activation of apoptosis signaling (27-29).

In the current study, we demonstrated the possible protective effect of *T. crispa* against Cisplatin-induced DNA damage as well as apoptosis in human epithelial MCF-10A cells. The data demonstrated that *T. crispa* significantly reduced Cisplatin-induced cytotoxicity in MCF-10A. Also, cellular DNA fragmentation ELISA analysis demonstrated that *T. crispa* significantly decreased the apoptotic activity induced by Cisplatin in MCF-10A. Consistently, the data also demonstrated that Cisplatin significantly up-regulated the
The combination significantly decreased caspase-3 and caspase-9 mRNA expression compared to cisplatin alone (***p<0.001). Analysis was performed by real time PCR using specific primer sequences (Table 2). Levels of caspase-3 and caspase-9 mRNA were normalized with β-actin and GAPDH (reference genes). Data are presented as means ± SD (n=6).

Several studies indicated that Cisplatin DNA damage induces a fall in the mitochondrial permeability transition; this in turn will cause mitochondrial rupture. Mitochondrial rupture releases cytochrome-c and procaspase-9 that bind to apoptotic protease activating factor 1 (Apaf-1) in an apoptosome complex, leading to the activation of caspase-9 (initiator protease) through the intrinsic apoptosis pathway. Activated caspase-9 induces other caspases to interact, resulting in activation of caspase-3 (effector protease) with the subsequent cleavage of key substrates (1-3, 19, 30). Caspase-3 and caspase-9 are key components in the mitochondrial apoptosis pathway (30-32).

Furthermore, our results displayed that Cisplatin significantly up-regulated the expression of NF-κB (p50/p65) while the combination treatment of both compounds significantly down-regulated the expression of NF-κB (p50/p65). NF-κB is a rapid response transcription factor in the cytoplasm and consists of 2 subunits of [NF-κBIA (p50) and NF-κB (p65) RELA] that are bound to an inhibitor protein called IκB. Several studies could show that Cisplatin increases degradation of IκB in a time-dependent manner and increases NF-κB binding activity (33-34). Eventually the freed NF-κB complex is able to translocate into the nucleus where it transactivates inflammatory genes such as Tumour Necrosis Factor.

**Fig. 5** The effect of *T. crispa* and cisplatin on the gene expression levels of caspase-3 (fig.5-A) and caspase-9 (fig.5-B) in MCF-10A: Shown are the data for MCF-10A cells treated with 18 µg/ml of cisplatin and/or 60 µg/ml of *T. crispa*. The combination significantly decreased caspase-3 and caspase-9 mRNA expression compared to cisplatin alone (***p<0.001). Analysis was performed by real time PCR using specific primer sequences (Table 2). Levels of caspase-3 and caspase-9 mRNA were normalized with β-actin and GAPDH (reference genes). Data are presented as means ± SD (n=6).
Factor-α(TNF-α), Tumour Growth Factor-α (TGF-α), Monocyte Chemo-attractant Protein-1 (MCP-1), Intercellular Adhesion Molecule-1 (ICAM-1) and hemeoxygenase-1. NF-κB coordinates the activation of a large network of chemokines and cytokines.(23) Recent evidence indicates that inflammation has an important role in the pathogenesis of Cisplatin-induced toxicity in normal tissues.(35) The results of this study clearly showed that the combination of T. crispa and Cisplatin significantly down-regulated the gene expression of TNF-α and ICAM-1 indicating less inflammation.

The fact that some antioxidants have already been demonstrated to be strong inhibitors of NF-κB activation may be one of the pathways by which they protect against cancers naturally, and also opens a promising avenue in the association between antioxidants and NF-κB activity in the development of therapeutic strategies.

5.0 Conclusion

Although the usage of chemotherapeutic drugs such as Cisplatin has been one of the most important methods for the treatment of cancer, their side effects however can be a major limitation for patient tolerance to cancer therapy. Our study showed that T. crispa ameliorated the effect of Cisplatin in non-cancerous epithelial cells by significantly decreasing the Cisplatin-induced cytotoxicity, genotoxicity, apoptosis and inflammation. Further animal studies and clinical trials are required to establish whether T. crispa can be usefully applied to cancer therapy with Cisplatin.

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References


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