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TINOSPORA CRISPA EXTRACT ENHANCES CISPLATIN-INDUCED APOPTOSIS IN TRIPLE NEGATIVE BREAST CANCER CELLS

Al-Rashidi RR[1], Ibahim MJ[2], Hamid Hasani NA[2], Froemming GRA*[2, 3]

[1]Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, University Teknologi MARA, Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia

[2]Faculty of Medicine, Universiti Teknologi MARA, Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia

[3]Institute of Pathology, Laboratory and Forensic Medicine (I-PPerForM), Universiti Teknologi MARA, Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia

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*Corresponding Author: Assoc. Prof Dr. Gabriele R.A. Froemming
Email: gabriele@salam.uitm.edu.my

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ABSTRACT

Tinospora crispa is a traditional medicinal plant with anti-cancer properties used in many South Asian countries. The aim of this study was to investigate the adjuvant properties of *T. crispa* on cisplatin treated Triple Negative Breast Cancer (TNBC) cell lines with regards to cell proliferation, apoptosis and NF-κB activation. MTT assay was performed to determine cell viability while flow cytometry and cellular DNA fragmentation ELISA was used to determine the cell death type and qPCR to investigate the mRNA expression levels of caspase 3, 8, 9 and NF-κB. *T. crispa,* on its own, moderately decreased the cell viability in TNBC cells with an IC_{50} of 66±3µg/ml and 60±4µg/ml in MDA-MB-231 and HCC1806 cells respectively. The main cause for *T. crispa* induced cell death was apoptosis with 92±1.25% and 89±0.95% in MDA-MB-231 and HCC1806 cells respectively. Furthermore, *T. crispa* significantly down-regulated the gene expression of NF-κB while significantly up-regulating the gene expression of caspases 3, 8 and 9 in both cancer cell lines. In conclusion, *T. crispa* showed moderate anti-proliferative effect on TNBC cells. However in combination with cisplatin, *T. crispa* acted as an excellent adjuvant in significantly down-regulating NF-κB gene expression followed by increased apoptosis in TNBC.

1.0 Introduction

Triple Negative Breast Cancer (TNBC) is a subtype of breast cancer that accounts for approximately 15% - 20% of breast cancer cases. TNBC is negative for expression of Progesterone Receptor (PR), Estrogen Receptor (ER) and ErbB2 [also known as Human Epidermal Growth Factor Receptor 2 (HER2)]. It is characterized by aggressive behavior, unique molecular profile, lack of targeted therapies and distinct pattern of metastasis [1]. Like other forms of breast cancer, TNBC is treated with surgery, radiation therapy, combined with chemotherapy if required. Current treatment regimens fail to restrain the aggressive behavior of TNBC in the majority of patients. A lack of targeted use of therapies coupled with a poor 5 year prognosis in TNBC patients is reflected in high mortality rates.

Several studies showed the efficacy of cisplatin in the treatment of TNBC [2-4]. Although the usage of chemotherapeutic drugs like cisplatin has been one of the most important methods for the treatment of cancer, their side effects can be a major restriction for patient tolerance to chemotherapy. Strategies to optimize the existing chemotherapy treatments have the potential to consolidate chemo-sensitivity and decrease their side effects. Recent studies suggested that a combined chemotherapy using plant extracts and cisplatin can improve the anti-proliferative activity of cisplatin in several types of cancers [5-7].

The inducing of apoptosis in cancerous cells alone, while sparing normal cells is one of the strategies of cancer therapy. Since apoptosis is a regulated process it does not release proteolytic enzymes into the tissue environment and, therefore, will not cause inflammation, destruction, or scarring and fibrosis of adjacent tissues [8, 9]. Therefore, modulating apoptosis may be valuable in the prevention of cancer treatment induced side effects.

Therefore screening of plant extracts or single compounds derived from plants is useful in finding adjuvant acting compounds. Current proof has emerged from different studies that suggest that natural products and compounds are practical in the prevention as well as in the treatment of cancer [5, 7, 10-14]. Cancer chemo-preventive drugs consist of a diverse group of compounds with diverse mechanisms of action, but, their decisive capability to induce apoptosis may well represent a unifying model for the mechanism of cancer treatment [8, 15].

Tinospora crispa is a well-known traditional medicinal plant used in Malaysia and other Asian countries. *T. crispa* reputed efficacies as an anti-proliferative agent against several types of cancers was confirmed by several studies [16-20]. In this study, we investigated the adjuvant properties of *T. crispa* when used with cisplatin on TNBC cells.

2.0 Materials and methods

2.1 Plant material

The plant raw materials of *T. crispa* were collected from a village in Chenor, Pahang in Malaysia. The identity of the plant was confirmed by a botanist in UPM and the folowing voucher specimen number was assigned: SK024. The stems of the plant were cut into small pieces (2 - 2.5 cm in length), oven dried and grounded into powder. The powder was soaked in methanol at room temperature, overnight 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 [9, 21]. For the preparation of the stock solution, the dried plant extract was reconstituted with methanol at a concentration of 10 mg/mL and then serially diluted in TNBC cell culture growth medium.

2.2 Chemicals and reagents

Cisplatin, dimethyl sulfoxide (DMSO), Roswell Park Media Institute (RPMI) 1640, Fetal Bovine Serum (FBS), Accutase, penicillin-streptomycin and MTT (3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were purchased from (Invitrogen, USA). Methanol of the highest pure grade was obtained from Merck, (Germany).

2.3 Cell culture

The MDA-MB-231 and HCC1806 cell lines were obtained from the American Type Culture Collection (USA). MDA-MB-231 and HCC1806 cell lines were grown in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37ºC with 5% CO_2 and 95% humidity until the culture was 70-80% confluent.

2.4 MTT assay

The effect of *T. crispa* and cisplatin on cell viability of TNBC cells; MDA-MB-231 and HCC1806, was determined using MTT assay. Cells ($2x10^4$) were cultured in a 96 well plate in 100 μL complete culture media and incubated overnight to allow for cell attachment. Cells were treated with different doses of *T. crispa* (0 to 100 μg/mL) and IC_{50} of cisplatin. Cells without treatment and cells treated with cisplatin only were used as negative and positive controls respectively. The cells were incubated at 37ºC in 5% CO_2 for 72 hours. 10 μL of MTT-PBS (5 mg/mL) was added to each well and further incubated for 4 hours at 37ºC in 5% CO_2 and 95% humidity. The medium was then removed and replaced with 100 μL of DMSO to solubilize the formed 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).

2.5 Flow cytometry

The Annexin V-FITC-apoptosis detection kit (Beckman Coulter, USA) was used to determine the mode of cell death. MDA-MB-231 and HCC1806 cells were cultured in a Petri dish with complete growth medium and then treated with different concentrations of *T. crispa*, cisplatin (IC_{50}) or combination of both for 72hrs. Cells were then washed twice with PBS, accutased and centrifuged at 500xg for 5 min at

4°C. The cell pellet was resuspended in ice-cold binding buffer to a concentration of $1x10^6$ cells/ml. Then 1 µL of Annexin V and 5 µL propidium iodide were added to the 100 µL of cell suspension. This was followed by the addition of 400 µL of ice-cold binding buffer. Cells were then incubated for 15 min on ice in the dark before analyzed by flow cytometry (Beckman-Coulter Inc., USA).

2.6 Cellular DNA fragmentation ELISA

The effect of *T. crispa* and cisplatin on cell apoptosis was investigated using DNA Fragmentation ELISA kit (Roche Molecular Biochemicals, USA). MDA-MB-231 and HCC1806 cells were adjusted to a cell density of $4x10^5$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% CO_2. Accutase was added to detach the cells, and then cells were centrifuged for 10 min at 250xg and re-suspended in BrdU free culture medium. The cells were adjusted to a cell density of $1x10^5$ 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 IC_{50} doses of *T. crispa* and cisplatin respectively the combination of both were added along with 100 µl/well of growth medium. The cells were further incubated for 72 h at 37°C in 5% CO_2 and 95% humidity. After that, the plate was centrifuged at 250xg for 10 min, media was removed and 200 µL/well of the lysing solution was added to lyse the cells.

Table 1: QPCR primer sequences

Gene	Primer sequence	Company
Caspase 3	F: 5'- GAGTGCTCGCAGCTCATACCT -3' R: 5'- CCTCACGGCCTGGGATTT -3'	Sigma, USA
Caspase 8	F: 5'- CTCCCCAAACTTGCTTTATG -3' R: 5'- AAGACCCCAGAGCATTGTTA -3'	Sigma, USA
Caspase 9	F: 5'- GAGTCAGGCTCTTCCTTTG -3' R: 5'- CCTCAAACTCTCAAGAGCAC -3'	Sigma, USA
NF-κBIA (p50)	F: 5'- AACCTGCAGCAGACTCCACT -3' R: 5'- ACACCAGGTCAGGATTTTGC -3'	Sigma, USA
NF-κB (p65) RelA	F:5' - TCAATGGCTACACAGGACCA -3' R:5' - CACTCTCACCTGGAAGCAGA -3'	Sigma, USA
GAPDH	F: 5' - TGCACCACCAACTGCTTAGC- 3' R: 5' - GGCATGGACTGTGGTCATGAG-3'	Sigma, USA
β-actin	F: 5' - CCTTCCTCCCTGGGCATGGA-3' R: 5 '- CACACGGAGTACTTGCGCTCA-3'	Sigma, USA

After an incubation time of 30 min at room temperature the plate was centrifuged again for 10 min at 250xg. 100 µL of the cell lysate 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 min. After the microtiter plate was cooled for 10 min at -20°C, the conjugate solution was added, and the plate was incubated for 90 min at room temperature. Cells were washed again, substrate solution was added, and the plate was incubated for 30 min at room temperature. Absorbance was measured at 370 nm. An increase in number of apoptotic cells is associated with an increase in absorbance.Acording to the instructions, a concentration of $1x10^4$ apoptotic cells per well reflects an absorbance reading (optical density reading) of 1.5.

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

MDA-MB-231 and HCC1806 cells were treated with IC_{50} dose of *T. crispa,* cisplatin or combination of both for 72 hours. Total RNA was extracted from treated MDA-MB-231 and HCC1806 TNBC 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 Nanodrop (NanoDrop ND-1000 spectrophotometer).

cDNA was produced using 1ng of total RNA in a 20 µL reaction with Sensiscript RT and Oligo-dT primers. Expression of caspase-3, caspase-8, caspase-9 and NF-κB were analyzed using real time PCR. A cocktail of 1x SYBR Green Supermix, 25µM forward and reverse primers, and 2 µL of cDNA template in a 25 µL volume was amplified using iQ™ 5 Real Time PCR detection system (Bio Rad, USA). Gene expression was normalised with β-actin and GAPDH as housekeeping genes and iQ™ 5 Real Time PCR software (Bio Rad, USA) for all samples. All the qPCR primer sequences (Table 1) were purchased from Sigma (USA).

2.8 Statistical analysis

All data are expressed as means ± S.D from at least three 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, IL). Differences at $p<0.05$ were considered as statistically significant.

Table 2 The IC_{50} values of *T. crispa* and cisplatin on MDA-MB-231 and HCC1806 after 72 hours of treatment.

Cell line	*T. crispa* IC_{50}	Cisplatin IC_{50}
MDA-MB-231 (cancer)	66 ± 3 µg/mL	12 ± 1 µg/mL
HCC1806 (cancer)	60 ± 4 µg/mL	8 ± 0.7 µg/mL

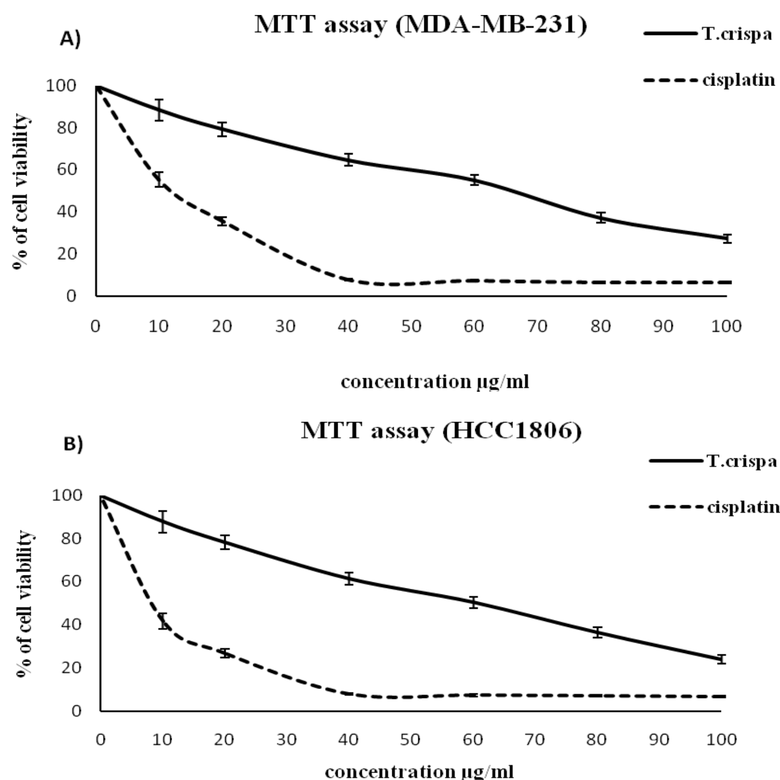

Fig.1: The anti-proliferative activity of *T. crispa* and cisplatin on TNBC cells: Shown are the data for MTT (MDA-MB-231 cells) (fig 1-A) and MTT (HCC1806 cells) (fig 1-B) incubated with various concentrations of *T. crispa* or cisplatin. The data represents the mean of 6 technical and 3 biological (n = 18) replicates ± S.D.
To identify the adjuvant effect of *T. crispa* on cisplatin treatment with regards to cell viability, the combination of the IC_{50} dose of *T. crispa* plus IC_{50} dose of cisplatin on TNBC cells MDA-MB-231 (66 µg/mL + 12 µg/mL) and HCC1806 (60 µg/mL + 8 µg/mL) were tested. The results obtained showed a significant increase in the percentage of cell death in the cancer cells compared to the use of *T. crispa* and cisplatin alone (p<0.001).

3.0 Results

3.1 *T. crispa* anti-proliferative effect on TNBC cells

To determine the anti-proliferative activity of *T. crispa* against TNBC cells (MDA-MB-231 and HCC1806 cells) TNBC cells were treated with different concentrations of *T. crispa* and cisplatin. *T. crispa* decreased the cell viability of MDA-MB-231 and HCC1806 in a dose dependent manner similar to the pattern of cisplatin. The IC_{50} values were obtained from the viability graphs and are listed in table 2. The IC_{50} values for MDA-MB-231 and HCC1806 were (66 ± 3 µg/mL) and (60 ± 4 µg/mL) respectively and it indicates a moderate anti-proliferative activity against both cancer cells

3.2 Flow cytometry

To determine the type of cell death induced by *T. crispa,* MDA-MB-231, and HCC1806 cells were treated with the IC_{50} doses of *T. crispa*, cisplatin or combination of both for 72 hours and then stained with annexin. Cells that are annexin V-FITC-positive, PI-negative (annexin V-FITC (+) PI (-)) were considered to be in an apoptotic stage, while annexin V-FITC-positive, PI-positive (annexin V-FITC (+) PI (+)) cells were considered to be necrotic. The percentage of apoptosis

calculated from the total cell death was 92.2±2% and 89.1±1% in MDA-MB-231 and HCC1806 cells respectively. Our results showed that the cell death induced by *T. crispa* and cisplatin was mainly due to apoptosis as presented in figure 3.

3.3 Cellular DNA fragmentation ELISA

To quantify and further investigate the contribution of apoptosis in the cell death induced by *T. crispa* and cisplatin, cellular DNA fragmentation ELISA was done. One characteristic of apoptosis is the selective fragmentation and packaging of nuclear DNA. The combination of *T. crispa* and cisplatin showed a significant increase of apoptotic activity in

MDA-MB-231 and HCC1806 ($p<0.001$) compared to single usage indicating a synergistic effect. These results are presented in figure 4.

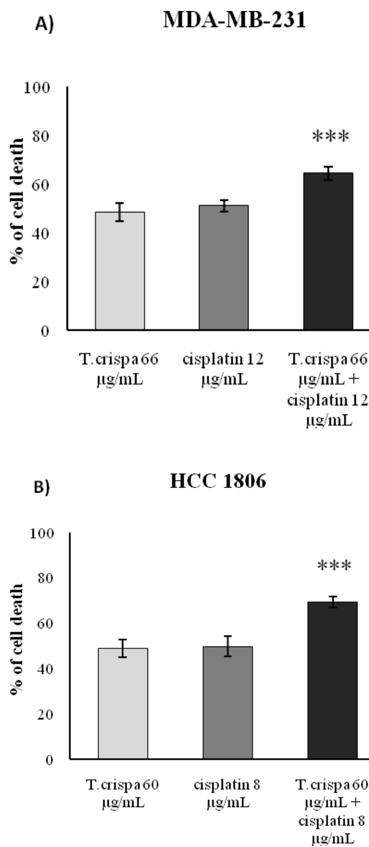

Fig.2: The anti-proliferative activity of *T. crispa* and cisplatin on TNBC cells: Shown are the data for MTT (MDA-MB-231 cells) (fig 2-A) and MTT (HCC1806 cells) (fig 2-B) incubated with IC_{50} doses of *T. crispa* and cisplatin. The data represents the mean of 6 technical and 3 biological (n = 18) replicates ± S.D.

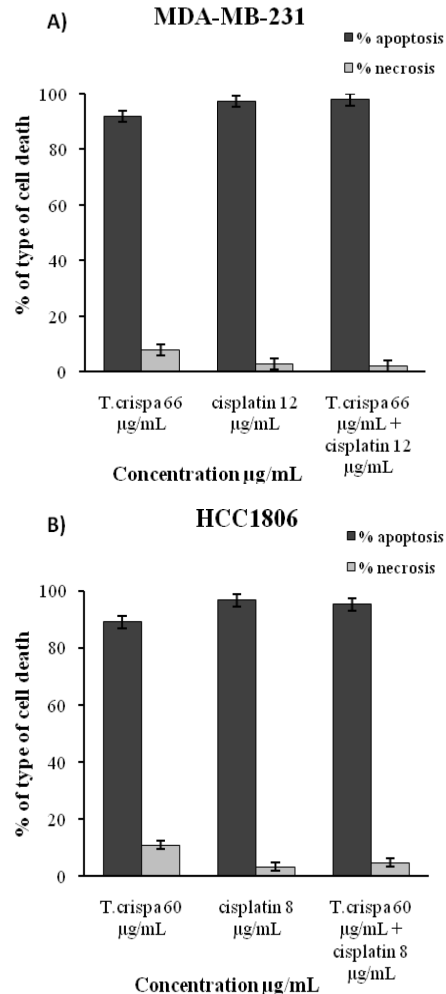

Fig.3: Type of cell death induced by *T. crispa*, cisplatin or combination of both in TNBC cell lines: Shown are the data for annexin V/PI (MDA-MB-231 cells) (fig 3-A) and annexin V/PI (HCC1806 cells) (fig 3-B). The cell death was mainly due to apoptosis in both cell lines. The data represents the mean of 3 biological (n = 3) replicates ± S.D.

3.4 Quantitative (Real-time) polymerase chain reaction (qPCR)

3.4.1 The effect of *T. crispa* on the expression levels of apoptosis related genes induced by cisplatin in MDA-MB-231 and HCC1806 cells

To understand the pathway involved in *T. crispa* and cisplatin-induced apoptosis in MDA-MB-231 and HCC1806 cells, the expression levels of caspase 3, 8 and 9 were investigated. Our results showed that *T. crispa* significantly up-regulated the gene expression of caspase 3, 8 and 9 similar to the results of cisplatin. Additionally, our results showed that treatment with cisplatin in the presence of *T. crispa* significantly up-regulated the gene expression of caspase 3, 8 and 9 in both cell lines as compared to single usage (figure 5).

This indicates a possible synergistic effect of *T. crispa* with cisplatin-induced apoptosis in TNBC cells. However, the increment of caspase 9 was significantly higher than the one of caspase 8 indicating a greater involvement of the intrinsic pathway of apoptosis.

3.4.2 The effect of *T. crispa* on the expression levels of NF-κB (p50/p65) genes induced by cisplatin in MDA-MB-231 and HCC1806 cells

Whether *T. crispa* inhibits the activation of the NF-κB (p50/p65) or not was assessed using qPCR. The term NF-κB generally refers in particular to p50-RelA [NF-κBIA (p50) - (p65) RelA] heterodimer and it is one of the most avidly forming dimers [22].

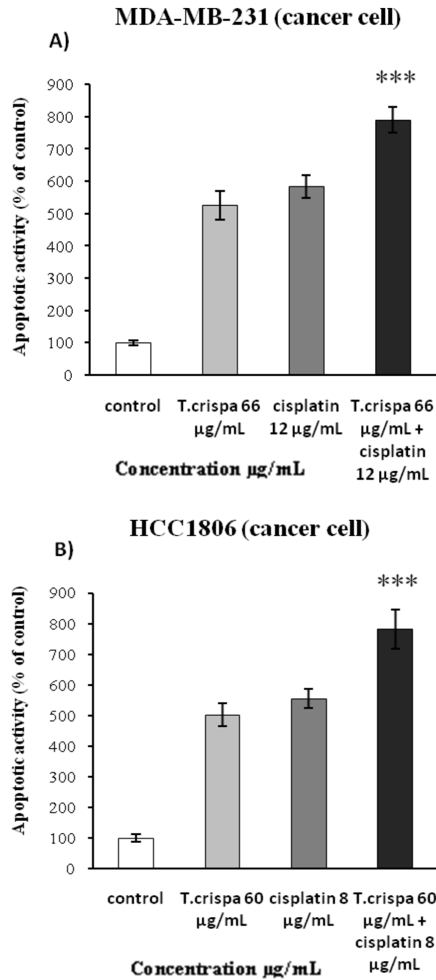

Fig.4: Apoptotic activity (represented as percentage of control) of *T. crispa* and cisplatin: Shown are the data for Cellular DNA fragmentation ELISA (MDA-MB-231 cells) (Fig.4-A) and Cellular DNA fragmentation ELISA (HCC1806 cells) (Fig.4-B). The data represents the mean of 6 technical and 3 biological (n = 18) replicates ± S.D. ***p<0.001

Cisplatin significantly up-regulated the gene expression of NF-κB subunits, p50 and p65. Unlike cisplatin, *T. crispa* caused no significant change in the mRNA expression levels of p50 and p65 proteins in both cancer cell lines. Aberrant regulation of NF-κB by several chemotherapeutic drugs including cisplatin has been linked to chemo-resistance in several types of cancer [23-26]. More interestingly, our results also showed that the addition of *T. crispa* to cisplatin treatment significantly reduced the gene expression of NF-κB subunits [NF-κBIA (p50) and NF-κB (p65) RelA] and thus sensitizing the cells to apoptosis.

4.0 Discussion

The search for novel anticancer agents is an on-going event. About 70% of the therapeutic drugs used nowadays are obtained from natural compounds and most of these compounds have cytotoxic and apoptotic properties [2, 13, 27-29].

T. crispa, a well-known traditional medicinal plant in Malaysia, is cytotoxic and has anti-proliferative properties in several types of cancers, including breast cancer [17-20].

Here we could show that *T. crispa* has a cytotoxic effect against MDA-MB-231 and HCC1806 TNBC cells lines. However, the IC_{50} values of *T. crispa* were significantly higher than the conventionally used chemotherapy drug cisplatin indicating lower potency for *T. crispa* as compared to cisplatin [32]. More interestingly, the combination of *T. crispa* and cisplatin significantly decreased the cell viability in TNBC cells compared to the single compounds indicating a synergistic effect in TNBC cells lines.

The flow cytometry data showed that the type of cell death TNBC cells underwent was apoptosis. Furthermore, the combination of *T. crispa* and cisplatin against cancer cells significantly increased apoptosis in both TNBC cell lines compared to single usage. These results were further confirmed by quantifying the apoptosis using cellular DNA fragmentation ELISA.

A successful anticancer drug should eradicate or debilitate cancer cells without causing any unnecessary damage to the normal cells. *T. crispa* has been shown to reduce cisplatin toxicity in normal breast epithelial cells [33] while in TNBC *T. crispa* increased cisplatin induced apoptotic cell death.
Crude extracts prepared from a variety of medicinal plants were demonstrated to possess the ability to trigger apoptotic activity and they can provide an efficient strategy for cancer therapy [34, 35].

The qPCR results for the mRNA expression levels of caspase-8, caspase-9 and caspase-3 showed a significant up-regulation when treating the MDA-MB-231 and HCC1806 cells with *T. crispa* or cisplatin as compared to control. Both cisplatin and *T. crispa* strongly reduced the cell viability in TNBC by triggering caspase-dependent apoptosis. Both compounds were able to trigger the upregulation of caspase-8 and caspase-9, which are implicated in the extrinsic and intrinsic apoptotic pathway, respectively. Moreover, the data presented clearly demonstrated that using a combination of *T. crispa* and cisplatin was significantly more effective in up-regulating the caspases mRNA level expression as compared to single usage. The increment of caspase-9 was significantly higher than that of caspase-8 indicating more involvement of the intrinsic pathway of apoptosis. Knowing which apoptotic

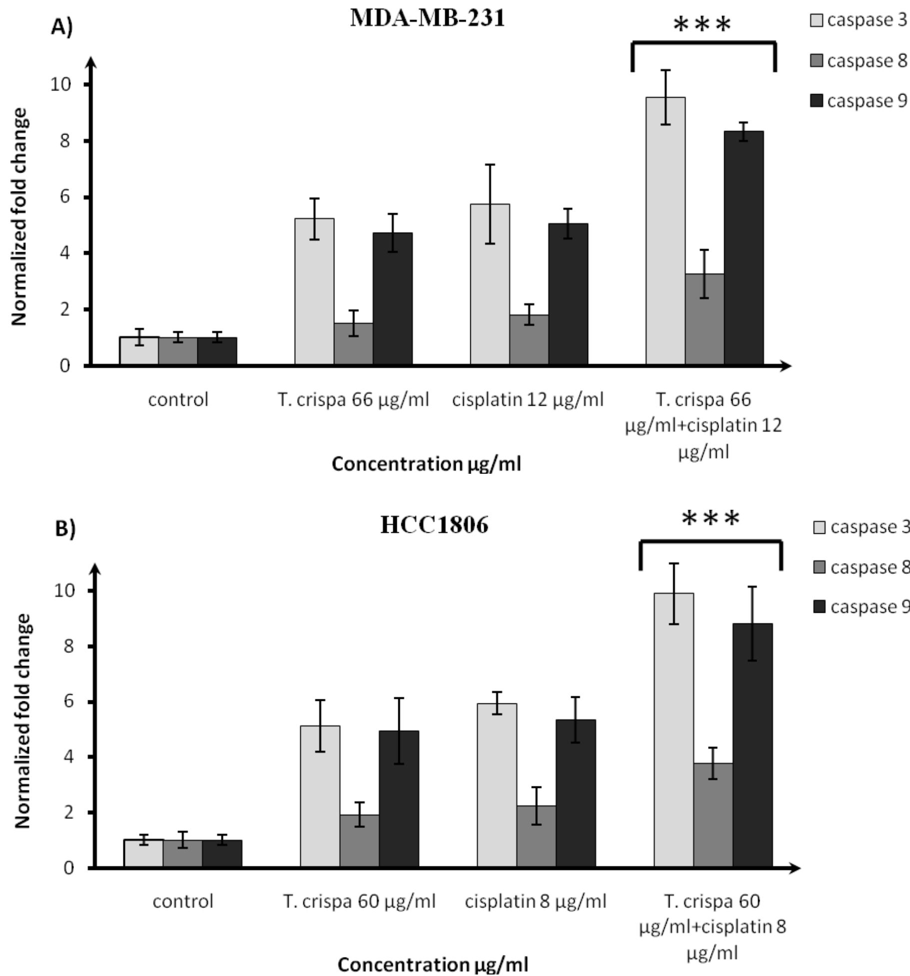

Fig.5: The effect of *T. crispa* and cisplatin on the expression levels of caspase 3, 8 and 9 in QPCR (MDA-MB-231 cells) (fig 5-A) and QPCR (HCC1806 cells) (fig 5-B): Shown are the data for both cells treated with IC_{50} doses of *T. crispa* and/or cisplatin. Analysis was performed by quantitative real time PCR using specific primer sequences (Table 1). Levels of caspase 3, 8 and 9 mRNA were normalized with β-actin and GAPDH (reference genes). Data are presented as means ± SD (*n*=6).

pathway is more involved in the cell death is crucial in understanding the molecular mechanism of action of *T. crispa*. It is possible that the role of *T. crispa*, in enhancing cisplatin-induced apoptosis, is restricted to the modulation of the apoptotic machinery and/or resistance to chemotherapy.

NF-κB is a transcription factor constitutively expressed in many cancer cell types that plays a fundamental role in numerous cellular mechanisms including cellular stress response, immunity, proliferation, motility and transformation. The term NF-κB usually refers particularly to p50/p65 heterodimer. The up-regulation of NF-κB signaling is a common feature of malignant disease and also in resistance to chemotherapy [23].

Genotoxic agents like cisplatin will induce nuclear DNA breaks. This will initiate signals that trigger the activation of nuclear-localized IKKγ. Once activated, IKKγ will translocate to the cytoplasm, where it activates IKK leading to phosphorylation and degradation of IκBα. IκBα is an inhibitory protein that binds to p50/p65 proteins and restrains the NF-κB complex in the cytoplasm. Once IκBα is degraded, the freed p50/p65 proteins will translocate to the nucleus and induce NF-κB dependent target genes [36]. Recent research studies have reported the role of several natural products in inhibition of the degradation of IκBα by different stimuli including chemotherapeutic agents [36, 37].

The here presented qPCR results showed a significant up-regulation for mRNA expression levels of p50 and p65 subunits of NF-κB in TNBC and normal cells following treatment with cisplatin. Unlike cisplatin, *T. crispa* did not show any significant change of p50 and p65 subunits expression of NF-κB in TNBC cells. More interestingly, our

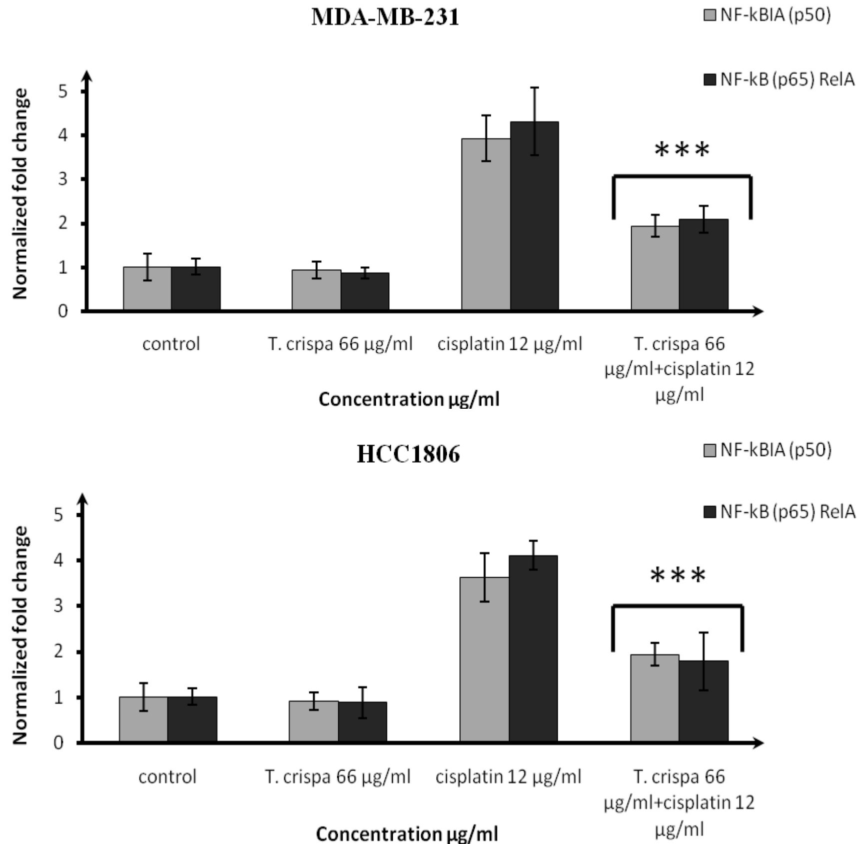

Fig.6: The effect of *T. crispa* and cisplatin on the expression levels of NF-κB (p65) RelA and NF-κBIA (p50) proteins in qPCR (MDA-MB-231 cells) (fig 6-A) and qPCR (HCC1806 cells) (fig 6-B): Shown are the data for both cells treated IC_{50} doses of *T. crispa* and/or cisplatin. Analysis was performed by real time PCR using specific primer sequences (Table 2). Levels of NF-κB (p65) RelA and NF-κBIA (p50) mRNA were normalized with β-actin and GAPDH (reference genes). Data are presented as means ± SD (*n*=6).

results showed a significant down-regulation for mRNA expression of p50 and p65 subunits of NF-κB in TNBC cells following treatment with combination of cisplatin and *T. crispa* as compared to cisplatin alone, this indicates that *T. crispa* protected the IκBα from cisplatin induced-degradation and thus prevented NF-κB activation since the single usage of *T. crispa* did not show any significant change. This also explains why the combination of *T. crispa* and cisplatin significantly down-regulated the mRNA expression levels p50 and p65 subunits of NF-κB in both TNBC cells.

An increasing body of evidence is revealing a tumor-suppressive and chemo-sensitizing role for NF-κB in cancer cell lines and NF-κB inhibitors are becoming prevalent in the treatment of cancer since NF-κB regulates many genes related to apoptosis like IAP proteins, RIP-1, TNFR2, Bcl-2 and Bcl-Xl [23, 38].

Understanding how *T. crispa* can alter the chemotherapeutic drug response will help shed light on the interaction of NF-κB inhibitors with current chemotherapeutic agents. In addition, it would open new avenues of how these inhibitors can be used in combination to create synergistic effects.

5.0 Conclusion

T. crispa showed anti-proliferative activity and when used in combination with cisplatin it was able to increase the potency of cisplatin to induce apoptosis in TNBC cell lines. *T. crispa* holds promise as a chemo-preventive agent when used in combination with other chemotherapeutic drugs. However, more work is needed to identify the molecular pathways through which *T. crispa* may function and to develop mechanism based biomarkers for evaluating *in vivo* and clinical studies outcome.

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