**Tinospora crispa** METHANOLIC CRUDE EXTRACT ACTIVATES APOPTOTIC PATHWAY OF INSULIN RESISTANT-HEPG2 CELL LINES BY IMPROVING THE INSULIN SENSITIVITY

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**ABSTRACT**

This study elucidates the growth inhibition of the insulin resistant-human hepatocellular carcinoma (IR-HepG2) cell lines after treating with *Tinospora crispa* methanolic crude extract. The IR-HepG2 cell lines were cultured in high glucose-insulin medium, subsequently established by the radiolabelled 2-deoxyglucose assay. The IR-HepG2 (negative control) and WRL68 (normal control) were treated with *T. crispa* methanolic crude extract and rosiglitazone malate (RM-positive control) respectively. The glucose uptake, downregulated IGF-1R and Bcl-2 protein, and apoptosis mechanism were determined using the immunoblotting method and transmission electron microscope (TEM). Glucose uptake was decreased in IR-HepG2 cell lines, while increased after treated with *T. crispa* methanolic crude extract and RM. The protein expressions of IR-HepG2 treated with *T. crispa* was significantly increased (p < 0.05) the insulin receptor (InsR), p-Akt, and GLUT4. IR-HepG2 cell lines stimulated with insulin and treated with *T. crispa* crude extract were found to activate the apoptosis pathway through the expression of caspases-3, 8, 9 and Bad. Ultimately, the growth of IR-HepG2 cell lines was inhibited via apoptosis after improving cell sensitivity towards insulin and its glucose uptake. Nevertheless, mechanism of *T. crispa* to induce the effects is still unclear either as a direct ligand or as a cofactor, and it requires further investigations.

**Key words:** *Tinospora crispa*, insulin resistance, insulin resistant-HepG2, apoptosis, cell culture, TEM

**INTRODUCTION**

Meta-analysis study showed that the type 2 diabetes is associated with liver cancer (Wang *et al.*, 2016). Diabetes increases the liver cancer risk by 43% out of 2061 patients (Davila *et al.*, 2005). Thus, effective treatment to overcome the diseases becomes a major challenge. One of the treatment strategies being conducted is by inhibiting the liver cancer cell through apoptosis (Nepal *et al.*, 2013). Improving the cell sensitivity towards insulin is believed to activate the apoptosis (Orgel & Steven, 2013). To prove the strategy, *Tinospora crispa* herb or locally known as Brotowali (southern region), Patawali (central region) and Akar Seruntun (northern region) (Zulkhairi *et al.*, 2008), was selected in this study.

The herb has been traditionally used to treat many diseases, including jaundice, fever, malaria, diabetes, rheumatism, inflammation, hypertension, fracture, urinary disorders, and maintaining good health (Ahmad *et al.*, 2016). But, the most intriguing findings, the herb possesses a broad range of pharmacological activities including anti-oxidant, anti-diabetic, anti-proliferation, anti-inflammation, immuno-modulatory and cardioprotective activities (Bisset & Nwaiwu, 1984; Choudhary *et al.*, 2010; Lam *et al.*, 2012; Chusri *et al.*, 2013). However, the effect of the herb in activating apoptosis by increasing cell sensitivity towards insulin is still unknown. Thus, the study aims to discover the effect by treating insulin resistant-human hepatocellular...
carcinoma (IR-HepG2) cell lines with *T. crispa* methanolic crude extract.

**MATERIALS AND METHODS**

**Preparation of methanol extract**

The fresh plant sample was sent to the Forest Research Institute of Malaysia (FRIM) labelled as SBD013/11, and identified as *Tinospora crispa* (L.) Hook. F. & Thompson. Selected matured and healthy cortex of *T. crispa* stems were dried, pulverised and kept tightly in a food grade plastic and stored at temperature 25°C. The methanolic extracts was obtained according to method by Zulkhairi *et al.* (2008) with minor modifications. Four hundred grams of powdered *T. crispa* sample was soaked in 2 L of methanol for overnight and filtered. The methanol residues were evaporated using a rotary evaporator (Heidolph™ Hei-Vap™). Dried extracts were kept on -80°C until further analysis.

**Establishment of insulin resistant (IR)-HepG2 cell lines**

HB-8065™ human hepatoma G2 (HepG2) and CL-48™ human normal liver (WRL68) cell lines were propagated in a 75 mL of Dulbecco’s modified Eagle’s medium until confluence (DMEM; Sigma Aldrich). The medium was supplemented with 10% foetal bovine serum (Invitrogen) and 2% penicillin-streptomycin (Invitrogen) prior to incubating at 5% CO₂, and 37°C. HepG2 cell lines were used to induce the insulin resistant cell according to Iyer *et al.* (2010) and Zhang *et al.* (2011) method with minor modifications. The culture medium was replaced with high glucose (4g/L) DMEM medium and incubated for another 36 hours. Subsequently, the cells were stimulated with 100 nM insulin and the medium was changed every three days for one month.

**Measurement of glucose uptake**

To confirm the establishment of insulin resistant state of the cells, glucose uptake activity in the cells was performed using radiolabelled 2-deoxyglucose based on method by Mohd-Radzaman *et al.* (2013). Approximately, 2 x 10⁶ cells/mL of IR-HepG2 and normal WRL68 cell lines were cultured in a 12 well, respectively. Cells were washed with serum-free DMEM thrice before incubating with the media for 2hrs and rewashed with Kerb-Ringer buffer (KRB) three times. The *T. crispa* extract was tested at concentrations of 12, 25, 50 and 100 μg/mL. Simultaneously, the rosiglitazone maleate (RM) was run as control. All treated cells were stimulated with or without 100 nM insulin and incubated for 1hr. Then, radiolabelled 2-deoxy-[1-3H]-glucose, 1μCi/mL (0.5 mL of radioactive tritium glucose diluted in 0.1 mM glucose solution) was added and incubated for 1hr. The cells were washed for three times with ice-cold KRB buffer and added with 0.1% SDS dissolved in PBS buffer (pH 7.4) for further solubilized. One millilitre sample was collected using a scintillation vial and mix with 15 mL of Ultima Gold LLT scintillation cocktail. The mixtures were vortex and the glucose uptake activity were measured using the liquid scintillation counter (Quantulus, 1220). All experiments were carried out in triplicate.

**Western blot analysis**

IR-HepG2 cells (10⁶ cells/well) were incubated in the 6-well plates containing the *T. crispa* extract at concentrations of 12, 25, 50 and 100 μg/mL for 24hrs. Cells were then stimulated with insulin for 5 mins prior to harvest. Western blotting procedures were followed based on Ismail *et al.* (2013) method with minor modifications. IR-HepG2 cells were mixed with 100 μL lysis buffer (HEPES, NP40, Glycerol and NaCl). The lysate was collected and incubated on ice for 10 mins before transferring into micro-centrifuge tubes and centrifuged for 10000 rpm at 4°C. Supernatant was then transferred into conical tube. The Laemli buffer (5:1) was added and heated at 100°C for 5 mins. Lysates were stored at -80°C prior use. For antibody detection, the cell lysate was loaded and separated on SDS-polyacrylamide gel followed by transferring to polyvinylidene fluoride (PVDF) membrane (Invitrogen, 0.22nm). Membranes were blocked with tris-buffered saline (TBS) containing 5% milk and incubated with primary antibody (1:1000, Santa Cruz, USA) for 15hrs at 4°C and followed by a secondary antibody (1:1000 Santa Cruz, USA) for 1hr at room temperature. The membrane was enhanced using a Chemiluminescent dye (Thermo Fisher Scientific) before transferred on x-ray films. The band intensity appeared from the film was evaluated using Image Studio™ Lite-Licor Ver 3.1 software in densitometry unit (DU) and normalized with β-actin protein as a loading control.

**Apoptosis micrographs analysis using transmission electron microscopy (TEM)**

At harvest the cell pellets were embedded in agarose gel before primarily fixed with 2.5% glutaraldehyde for 5hrs at 4°C, and washed with 0.1 M sodium phosphate buffer (pH 7.2) twice. The pallet was re-fixed with 1% osmium tetroxide. Samples were then dehydrated (three-time changes with 30%, 50%, 70%, 90% and 100% ethanol), infiltrated (with propylene oxide and resin mixture in 1:1 ratio for 24hrs), embedded into beam capsule and polymerized in the oven for 60°C at 24-48hrs. Semi-thin section (1μm) were cut using ultramicrotome Leica EM UC7 and stained with...
toluidine blue to ensure the cells position. Ultrathin sectioning (700–100nm) was performed and mounted on 200nm mesh grids. The grid-section was then stained with 2% uranyl acetate for 1min at room temperature. Sections were observed, and images were captured under Hitachi (H-7650) electron microscope, at 80KV. The images were then transferred to Microsoft photo software for analysis.

Statistical analysis
Data were analysed statistically by SPSS software version 18. One-way analysis of variance (ANOVA) was used followed by Tukey’s post-hoc test. Data were presented as mean ± standard error mean (SEM). Statistically different means were recognized at \( p < 0.05 \).

RESULTS AND DISCUSSION
Establishment of insulin resistance (IR)-HepG2 cell lines
This study was successfully established insulin resistant-HepG2 cell lines by cultivating the HepG2 cells in high glucose medium (4g/L, DMEM) and stimulated by 100nM insulin for a month. After insulin resistance establishment procedure, the glucose uptake result of IR-HepG2 was measured low compared to untreated HepG2 by 0.5-fold decrease. IR-HepG2 cell lines were also exhibited a significant low in glucose uptake compared to WRL68 by 0.7-fold decrease. In the meantime, WRL68, a normal control liver cells shows a significant increase in glucose uptake after treated with \( T. crispa \) (0.4-fold) and even better toward the stimulation of insulin (0.9-fold). The entire of glucose uptake results can be seen in Figure 1. A comparable result of 100μg/mL of \( T. crispa \) extract and 100μg/mL RM (known anti-diabetic drug) on IR-HepG2 was exhibited an elevation by 2.5-fold and 1.5-fold, respectively. On the other hand, after insulin stimulation the elevation was significantly increased in both treatment by 2.9-fold and 2.5-fold, respectively. Glucose uptake in IR-HepG2 treated with \( T. crispa \) was elevated more in insulin stimulation compared to non-stimulated one. Overall findings have shown the ability of \( T. crispa \) treatment to improve glucose uptake in a dose

![Fig. 1. The histogram represents the percentage of 2-deoxy-[1-3H]-glucose uptake against rosiglitazone maleate (RM) and \( Tinospora crispa \) in normal liver (WRL68), liver cancer (HepG2) and insulin resistance (IR)-HepG2, with and without the presence of insulin stimulation (100nM). The value presented are mean ± standard error mean (SEM) from triplicates. Statistically significant data given are the comparison in between; (*) \( p < 0.05 \) IR-HepG2 vs. treatments, (**) \( p < 0.05 \) between untreated HepG2 vs. IR-HepG2 and (*** \( p < 0.05 \) between WRL68 vs. WRL68 with insulin stimulation and \( Tinospora crispa \) extract (100μg/mL).]
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Glucose uptake and apoptosis

Our results showed that protein expression was related to the glucose uptake (Figure 2a). Insulin receptor (InsR) band of IR-HepG2 (Figure 2a) treated with T. crispa were gradually bolder and thicker in every treatment compared to the InsR of untreated IR-HepG2 cancer cell lines which was thin and slender. The findings were identical in glucose uptake results in Figure 1. The IR-HepG2 treated with T. crispa showed higher glucose uptake compared to the untreated IR-HepG2. Results in phosphor-Akt antibody (Figure 2d) and Py-20 (Figure 2e) depicted the phosphorylation of Akt was dependant manner. This finding suggested that T. crispa treatment complemented with insulin equivalents to current anti-diabetic drug (RM) in treating IR-HepG2 cell lines.

<table>
<thead>
<tr>
<th>Protein</th>
<th>T. crispa (μg/mL) treatment on IR-HepG2 (Insulin resistant-HepG2)</th>
<th>Untreated IR-HepG2 (Negative Control)</th>
<th>T. crispa (μg/mL) treatment on WRL68 (Normal liver cells)</th>
<th>Untreated WRL68 (Normal Control)</th>
<th>Doxo (0.5mM) RM (100μg/mL)</th>
<th>Doxo (0.5mM) RM (100μg/mL)</th>
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</thead>
<tbody>
<tr>
<td>a) Insulin Receptor (InsR)</td>
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<td>b) GLUT-4</td>
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<td>c) GAPDH</td>
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<td>d) p-Akt</td>
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<td>e) Py-20</td>
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Fig. 2. Protein bands from Western blotting analysis involved in insulin related cell signalling of IR-HepG2 cancer cells lines and WRL68 normal liver cells were treated with Tinospora crispa in different concentration (6, 12, 25, 50 and 100μg/mL), rosiglitazone maleate (RM 100μg/mL) as anti-diabetic control drug and Doxorubicin (0.5mM) as an anti-proliferative control drug. The blots were incubated with primary antibodies overnight followed by secondary antibodies for 1hr. ß-actin was used as a protein loading control. The study was carried out in triplicates.
induced by the *T. crispa* in both HepG2 and WRL68 cells, leading to activation and upregulation of GLUT4 (Figure 2b) in both cells. Our results suggested that, *T. crispa* recruited the PI3K/Akt pathways via activation of insulin receptor, thus contributed to glucose uptake and cell proliferation in both cells.

The untreated IR-HepG2 demonstrated lower expression of Akt protein band (Figure 2d) compared to the IR-HepG2 treated with *T. crispa*, hence become an indicator of low glucose uptake and GLUT-4 downregulation in IR-HepG2. Insulin growth factor-1 receptor (IGF-1R) mediated protection of IR-HepG2 cell lines from apoptosis, thus the activation of IGF-1R led to increase cell proliferation and differentiation (Enguita-Germán et al., 2014). It has been rectified in Figure 2, where IGF-1R protein band (Figure 2f) is thicker and bolder in untreated IR-HepG2, yet the band’s intensities were gradually decreased after treated by both *T. crispa* and doxorubicin. Validation of downregulation in IGF-1R was made through phosphorylation-IGF-1R (p-IGF-1R) and the results were identical (Figure 2g). The anti-apoptosis activities of Bcl-2 protein in untreated IR-HepG2 were higher as seen in Figure 2h. The presence of high band intensities in IGF-1R and Bcl-2 anti-apoptotic proteins may explain the remarkable exertion of the HepG2 cells protecting itself from apoptosis. The efficacy of *T. crispa* as anti-proliferative agent was demonstrated in the presence of increased pro-apoptosis protein expressions, including caspases 8, 9, 3 and Bad (Figure 2i to 2l). These results indicated that *T. crispa* treatment promotes apoptosis in IR-HepG2 in a dose-dependent manner. Furthermore, Western blot analysis demonstrated that *T. crispa* downregulated Bcl-2 expression (Figure 2h), especially in 100μg/mL of extract, and upregulated Bad, caspase-9, caspase-8 and caspase-3 protein expressions (Figure 2i - 2l) in a dose-dependent manner. The mechanism of action in the glucose uptake by the *T. crispa* treated cell was concordance to a study conducted by Shen et al. (2012). HepG2 treated with sequoyitol herb increased the InsR sensitivity via insulin receptor substrate-1 (IRS-1) and Akt pathway (Shen et al., 2012).

**Apoptosis micrographs analysis using TEM**

Transmission electron microscopy (TEM) is one of the best tools to demonstrate the hallmarks of morphological apoptosis occurred in a cell. The morphology can be observed in the forms of chromatin condensation, nucleus fragmentation, shrinkage and cell blebbing (Ziegler & Groscurth, 2004). Morphology of normal liver cells WRL68 (normal control) and IR-HepG2 cell lines (negative control) was appeared intact (Figure 3A and 3E). Chromatin appeared intact, thickening of the nuclear membrane and nucleus irregularities were only observed in IR-HepG2 treated with *T. crispa* (Figure 3F and 3G) and doxorubicin (Figure 3H). IR-HepG2 treated with 50μg/mL *T. crispa* also exhibited cytoplasm autophagosomes (black arrow). IR-HepG2 cell morphology treated with *T. crispa* turned pleomorphism with plasma membrane blebbing (cb) with more features of necrosis (Figure 3F and 3G). Prominent nucleoli were an indication of reparative or damaged cells, and clearly seen in both WRL68 and IR-HepG2 treated with *T. crispa* and doxorubicin. Few nuclear grooves were also observed (Figure 3F and 3H). Other than blebs on cytoplasm, large vacuolation can be spotted in the cytoplasm of IR-HepG2 cells. The same condition occurred in WRL68 where more obvious blebbing was appeared (Figure 3B and 3C). Blebs are balloon-like vesical shape of plasma membrane found in the injury cell cytoplasm (Ziegler & Groscurth, 2004). Such normal blebs followed by shrinkages have been observed in the healthy normal liver; (Figure 3B and 3C). These results confirmed that IR-HepG2 treated with *T. crispa* methanolic crude extract exhibited apoptosis morphology in IR-HepG2 cells in a dose-dependent manner. The *T. crispa* extract possessed anti-proliferative effects and managed to induce apoptosis in IR-HepG2, thus exhibited the apoptosis morphologies as described by Ziegler and Groscurth (2004) and Elmore, (2007). Those morphologies are including the blebs formation, nucleus degenerated, cytoplasm disintegrated and auto-phagosome.

**Schematic illustration of *T. crispa* signalling pathway**

Results showed that methanolic crude extract of *T. crispa* triggered the insulin signalling pathway via several signalling proteins such as Akt, GLUT4 and GADPH. Figure 4 summarised the effects of *T. crispa* extract on IR-HepG2 cells. InsR and IGF-1R were involved in different cellular processes, including the cell proliferation, glucose metabolism, survival and differentiation. Tyrosine kinase (PY20) on the InsR plays a major role in proliferation and transformation of glucose metabolism. However, our finding in the glycolysis of IR-HepG2 has been altered was concordance with Gao et al. (2015), which using the 4-hydroxyisoleucine to improve IR-HepG2 by stimulated IRS-1 and GLUT-4 expression. Previous research was also unveiled the role of InsR and IGF-1R in tumorigenesis and treatment resistance (Emily, 2010; Vigneri et al., 2015). To control the IR-HepG2 growth, IGF-1R must be decelerated.
Fig. 3. Electron micrograph of the normal liver; WRL68 (A, B, C, D) and cancer liver cells; HepG2 (E, F, G, and H). Apoptosis occurred in both WRL68 and HepG2 cell treated with *Tinospora crispa* (100μg/mL) and doxorubicin (0.5mM) accordingly. Noted the typical apoptotic features of condensed chromatin, irregular nuclear outline, cells shrinkage and blebbing formations. Nucleolus (n), Nucleus (Nc), heterochromatin (he), euchromatin (ec), mitochondria (m), ribosome (r). TEM images depict ultrastructure of autophagosome in IR-HepG2 cells treated with *Tinospora crispa* and Doxo. Few autophagy vesicle with typical layer membrane containing organelle remnants was highlighted by white arrows. Bar = 1 μm.
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Fig. 4. Tinospora crispa extract as extracellular and intracellular stimuli improved the insulin resistance condition simultaneously restored the glucose uptake and induce apoptosis in IR-HepG2 cell lines.

CONCLUSIONS

*T. crispa* methanolic crude extract induces glucose uptake and apoptosis in IR-HepG2 cell lines. Crosstalk between phosphorylation of InsR and dephosphorylating of IGF-1R by *T. crispa* is believed to initiate the apoptosis and inhibited the cancer cell lines (HepG2), but nontoxic to the normal liver cells (WRL68). Therefore, *T. crispa* crude extract may possess a hepatoprotective effect as well act as an antiproliferative agent in inhibiting liver cancer cells. High sensitivity of the IR-HepG2 cell towards insulin with the facilitation of *T. crispa* extract may activate the apoptosis pathway in the cells and inhibit the growth of cancer cells. Nonetheless, the function of the *T. crispa* crude extract either as a direct ligand or as a cofactor remains unclear.

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