Immunomodulatory Effect and an Intervention of TNF Signalling Leading to Apoptotic and Cell Cycle Arrest on ORL-204 Oral Cancer Cells by Tiger Milk Mushroom, *Lignosus rhinocerus*

Running head: Apoptosis and Immunomodulation Activities of *L. rhinocerus*

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SUMMARY

Research background. Tiger milk mushroom (*Lignosus rhinocerus*) is a medicinal mushroom that is geographically distributed in the region of South China, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea. Consumption of its sclerotium has been reported to treat various ailments. However, its anticancer potential towards oral cancer cell lines is yet to be discovered considering its traditional method of consumption by biting/chewing of the sclerotium.

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**Experimental approach.** Mushroom sclerotial powder of cultivar TM02® was extracted and fractionated by a Sephadex G-50 chromatographic column prior to cytotoxicity testing against a panel of human oral cancer cell lines. The capability of the identified bioactive fraction in regulating several molecules associated with its TNF pathway was investigated.

**Results and conclusions.** MTT proliferation assay indicated that ORL-48 (derived from gingiva), ORL-188 (derived from the tongue), and ORL-204 (derived from buccal mucosa) were inhibited by *L. rhinocerus* sclerotial cold water extract and its high-molecular-mass fraction (HMM) in varying degree with ORL-204 being most affected. Hence, HMM treatment on ORL-204 was further investigated. HMM induced apoptosis and G0/G1-phase cell cycle arrest through caspase-3/7 cleavage. Activities of MIP2 and COX-2 were downregulated by 0.2- and 4.6-fold respectively in the HMM-treated ORL-204 cells.

**Novelty and scientific contribution.** Using ORL-204, it revealed that HMM may have intervened via the TNF pathway at various network sites in its manifestation as a potential dietary compound for cancer prevention and natural adjunct therapeutic to conventional cancer treatment.

**Key words:** *Lignosus rhinocerus*, oral cancer, apoptosis, cell cycle, COX-2, MIP2

**INTRODUCTION**

*Lignosus rhinocerus* or tiger milk mushroom (also known locally as ‘cendawan susu harimau’) has well-recorded medicinal values (1). Its sclerotium is traditionally used as a health tonic or treatment regime for asthma, bronchitis, various cancer ailments as well as discomforts caused by fright, fever, cough, vomiting, and injury (2,3). It is being consumed in the form of decoction, in a betel quid, and other preparation method where the sclerotium is pounded with raw rice, infused, and subsequently taken as a drink (4,5). A technique mimicking cold water extraction has been described by Chan (6) where the sclerotium is grated on a hard surface such as granite plate with some water and the resulting mixture is further diluted with water before consumed. There is also a practice of biting/chewing of the sclerotium by local indigenous communities during their journeys in the wild (7).

Previous omics studies reported the presence of lectins, fungal immunomodulatory proteins, superoxide dismutase, aegerolysin and laccases in *L. rhinocerus* that could be involved in various bioactivities, including immunomodulatory properties (8-10) which plays a pivotal role in diseases such as cancer. In fact, the anticancer properties of *L. rhinocerus* in numerous cell lines was previously
reported. A polysaccharide-protein complex from *L. rhinocerus* sclerotium has been shown to inhibit the growth of several leukemic cell lines induced by a G1-phase cell cycle arrest (11) while a cold aqueous extract preparation derived from cultivar KUM61075 exhibited cytotoxicity against a panel of human cancer cell lines. The cytotoxic component(s) was speculated to be thermo-labile, water-soluble protein/peptide(s) (12). On the other hand, Lee *et al.* (13) demonstrated the anticancer properties of a sclerotial cold water extract from TM02® cultivar; a protein- and carbohydrate-rich extract, against breast cancer MCF7 and lung cancer A549 cell lines. The efficacy of its molecular weight fractions and a partially purified cytotoxic serine protease-like protein against MCF-7 cells via a cross-talk in between intrinsic and extrinsic apoptotic route has also been reported (14). However, despite the growing scientific data of beneficial therapeutic effects of *L. rhinocerus* against various cancer cell lines, its anticancer potential towards oral cancer cell lines remains unknown. In this study, we investigated the anticancer activity of TM02® sclerotial extracts on a panel of human oral cancer cell lines and its possible mode of action.

Oral cancer is selected as it is one of the more common cancer in the world with estimated 354,864 new cases diagnosed, and 177,384 deaths reported in the year 2018 (15). Furthermore, tiger milk mushroom has been eaten by way of chewing and kept in mouth for a considerable amount of time, prior to swallowing. Hence, it is intriguing to find out its cytotoxicity in the oral cavity environment. More than 90% of oral cancer that arises from the lips and oral cavity are squamous cell carcinoma (16). It is believed that oral squamous cell carcinoma (OSCC) develops through stages, from increasing severity of histological changes of premalignant lesions to malignancy. OSCC is life-threatening and with a mere five-year survival rate for Stages 3 and 4. Early signs of oral cancer often go unnoticed and have been frequently discovered during routine dental examinations. Many cases of oral cancer may have advanced to an untreatable stage where the cancer cells have become aggressive and unresponsive to therapeutic drugs (17,18). In general practice, oral cancer is treated with either surgery, radiotherapy, and/or chemotherapy. The treatment outcomes may include disease recurrence and post-treatment morbidity owing to the non-specific damages of these treatments to function and healthy cells. Several determining factors that may increase the risk of cancer have been identified and these include massive exposure to chemical carcinogens such as tobacco and alcohol, solar ultraviolet radiation by excessive sun exposure to the lips, human papillomavirus infection, and a weakened immune system (19). Therefore, research that focused on natural immunomodulators to impede side effects of cytotoxic drugs has been gaining limelight in recent years. And often, natural compounds with simultaneous targeting of cancer pathways
may result in efficient and selective killing of cancer cells which could be an added advantage for treatment of the disease (20,21). Thus, it is of tremendous interest if the anticancer properties reported for this medicinal mushroom is also effective for oral cancer.

MATERIALS AND METHODS

Extraction and fractionation of TM02® sclerotial powder

The freeze-dried sclerotial powder TM02® (Reg no. MAL 11035004TC) was provided by LiGNO™ Biotech Sdn. Bhd. (Balakong Jaya, Selangor). Preclinical toxicological study determined that the product was not associated with any toxicity concerns. No-observed adverse-effect level dose was more than 1,000 mg/kg. The powder also did not cause detectable adverse effect on rats’ fertility, teratogenic, and genotoxicity effects (22). Hot water, cold water and methanol extractions were carried out in a mass to volume ratio of 1:20 (g/mL) as per described earlier (23). Sclerotial cold water extract of TM02® was further fractionated by Sephadex® G-50 (fine) (Sigma-Aldrich, St. Louis, Missouri, USA) gel filtration chromatography column equilibrated with 0.05 M ammonium acetate (Sigma-Aldrich, St. Louis, Missouri, USA) buffer. Eluted fractions were subsequently grouped based on their molecular masses.

Cell culture and maintenance

ORL-48, ORL-204, and ORL-188 oral cancer cell lines isolated from respective gingiva (gum), buccal mucosa (lining of the cheeks and back of the lips), and tongue were obtained from Cancer Research Malaysia (Subang Jaya, Selangor). These cells were established from surgically resected specimens obtained from untreated primary human oral squamous cell carcinomas of the oral cavity as in vitro models to study a disease prevalent in Asia. Their growth characteristics, epithelial origin and molecular alterations were previously characterized (24,25). Genetic information and clinical data associated with these ORL cell lines are available at https://genipac.cancerresearch.my/ (26). ORL-48, ORL-204, and ORL-188 cells were cultured and maintained in DMEM/F-12 media (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10 % fetal bovine serum (Nacalai Tesque Inc., Kyoto, Japan) and 0.1 % penicillin-streptomycin (Sigma-Aldrich, St. Louis, Missouri, USA) at 37 °C, 5 % CO₂.

MTT cytotoxicity assay
MTT assay was used to determine the anti-proliferative activity of the extracts. The yellow tetrazolium dye will be reduced to purple crystalline formazan in metabolically active viable cells by NAD(P)H-dependent cellular oxidoreductases. ORL cell lines were seeded in monolayer and were allowed to adhere overnight prior to treatment with TM02® samples at different concentrations from 31.25 µg/mL to 1000 µg/mL. Following 72 h of incubation, 20 µL of 5 mg/mL MTT solution (Calbiochem, San Diego, California, USA) in phosphate buffered saline (Oxoid, Basingstoke, England) was added to each well. The plate was then incubated for 4 h at 37 °C to promote the formation of purple formazan crystals. All the solutions were then aspirated and 200 µL of DMSO (Sigma-Aldrich, St. Louis, Missouri, USA) was added in order to dissolve the attached formazan crystals. Absorbance was read at 570 nm after it was incubated for 10 to 30 min in the dark. Cell viability (%) was calculated and plotted against the extract concentration curve.

Caspase activity measurement

Caspase-3/7, -8 and -9 activities were measured using respective Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 Assay Systems (Promega, Fitchburg, Wisconsin, USA) according to manufacturer’s protocol. Cells were seeded in monolayer into a 96-well white plate and treated with high-molecular-mass fractions of TM02® cold water extract (HMM) at 75 µg/mL for 24, 48, and 72 h. After treatment, luminescent signal which was proportional to caspase activity was measured an hour after the addition of Caspase-Glo® Reagent; which relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), to the post-treated cells in 1:1 ratio.

Flow cytometry analysis of cell cycle

Cell cycle distribution of treated cells was quantitated using Muse™ Cell Cycle Kit (Millipore, Burlington, Massachusetts, USA) according to manufacturer’s protocol. In brief, cells were treated with HMM at 40 and 250 µg/mL (IC75) for 72 h prior to 70 % ethanol fixation and staining with Muse™ Cell Cycle Reagent; a nuclear DNA stain containing propidium iodide, prior to analysis on Muse™ Cell Analyzer SN. No 7200150631 (Millipore, Burlington, Massachusetts, USA). The percentages of cells in the G0/G1-, S-, and G2/M-cell cycle phases which differ in DNA content were quantitated by fluorescence-activated cell sorting (FACS) analysis with the configuration of 532 nm green laser line and three detection channels.
ELISA assay

Selected modulators (TIMP1, MIP2) were quantitated using ELISA kit (Elabscience, Wuhan, China) according to manufacturer’s protocol. In brief, cells were treated with HMM for 72 h at 10 µg/mL (IC_{25}) and 40 µg/mL. A total of 100 μL of standard or sample (the collected spent media from treated cells) was then added into each pre-coated well and incubated for 2.5 h at 37 °C to combine with the specific antibody. Specific biotinylated detection antibody was then added into each well, incubated for 1 h at 37 °C prior to the successive addition of Avidin-Horseradish Peroxidase conjugate for 1 h incubation at 37 °C after washing three times. Unbound components were washed off and substrate solution was added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution. Absorbance was measured at 450 nm using Epoch Microplate Spectrophotometer SN. No 255746 (Winooski, Vermont, USA).

Cyclooxygenase (COX) assay

COX activity assay kit (Fluorometric) was purchased from Abcam, Cambridge, UK to detect the peroxidase activity of COX. A fresh set of standards was prepared and supernatant from treated cells (HMM treatment for 72h at 40 µg/mL) were collected according to manufacturer’s protocol. Samples were kept on ice for downstream procedures. Standard wells and reaction wells of samples and positive control were prepared and 10 µL of diluted arachidonic acid/NaOH Solution was added into each reaction well followed by the addition of arachidonic acid. Fluorescence was measured (Ex/Em=535/587 nm) in a kinetic mode once every 15 s for 30 min.

Statistical analysis

SPSS Statistics 21.0 (27) with one-way ANOVA followed by LSD’s post hoc test for multiple comparisons was used to compare the mean values. A p value of less than 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

In this study, pulverized TM02® was extracted with hot water, cold water and methanol to coerce its carbohydrates, proteins, and secondary metabolites, respectively. The yield and composition of these extracts were previously reported by our lab (23). Hot water extract (HWE) consisted almost entirely of
carbohydrates with 56% of α-glucans while cold water extract (CWE) retained most of the extractable proteins as extraction at low temperature of 4 °C had prevented the excessive degradation of thermolabile constituents including proteins and peptides. The major constituent of carbohydrates in CWE is mainly glucose which makes up the glucans in linear polysaccharides with 1,4-linkage. Meanwhile, methanol extract (ME) consisted mainly of secondary metabolites (terpenoids) with no detectable level of proteins (23, 28).

The ORL-48, ORL-204, and ORL-188 oral squamous cell carcinoma (OSCC) cell lines were selected as they represent some of the most common areas for oral cancer with 100% orthotopic take rate and highly hyperplastic (24). These cells were treated with HWE, CWE and ME from 31.25 μg/mL to 1000 μg/mL for 72 h. Cytotoxicity effect of the crude extracts was then measured with MTT assay. The reduction in quantity of cells indicates inhibition of cell growth and cells’ drug sensitivity is further specified as the drug concentration needed to reach 50% inhibition of cell growth (IC50). ORL-48 and ORL-204 were more responsive toward CWE (Fig. 1) and the observed differences could be attributed to genetic predisposition resulting from site variation as ORL-48 and ORL-204 were isolated from gingiva and buccal mucosa while ORL-188 was from tongue (24).

Owing to its anti-proliferative potential, CWE was further fractionated via Sephadex® G-50 column to high-, medium- and low-molecular mass (termed HMM (>30 kDa), MMM (7–30 kDa) and LMM (<7 kDa), respectively). In our previous study, we reported that HMM contains the most amount of carbohydrates and proteins, with no detectable level of β-glucans, suggesting the existence of polysaccharide-protein complexes in the fraction. Our group reported that HMM contains the 1,3,6-GlcC glycosidic linkage in the prospective polysaccharide-protein complexes (28). On the other hand, LMM has a lower amount of carbohydrates with very little proteins, but higher number of secondary metabolites such as phenolics and terpenoids. MMM has moderate amount of the macromolecules and secondary metabolites. It contained highly branched glucans with mixed linkages including 1,4,6-GlcC, 1,3,6-GlcC and 1,2,4,6-GlcC (28, 29).

HMM, MMM, and LMM were tested on the OSCC cell lines with concentrations ranging from 16 μg/mL to 500 μg/mL for 72 h (Table 1) where a lower range of concentration was selected as compared to the crude extracts’ as stronger toxicity effect was expected. The range of IC50 values for HMM was determined to be from 40 to 115 μg/mL while MMM was from 125 to 175 μg/mL and LMM exceeded 400 μg/mL. HMM was found to be more cytotoxic on OSCC cell lines, specifically ORL-204, which was
originated from buccal mucosa cancer patient. Further testing of these extracts and fractions on an in-house isolated primary human fibroblast culture indicated that they are not toxic to normal tissues (Table 1). As HMM mainly consists of carbohydrates and proteins, the present result suggests that the bioactive component(s) responsible for its cytotoxic activity could be of proteoglycan nature and/or carbohydrate-protein complex derivatives.

We further determined if HMM induced apoptosis in ORL-204 by investigating the regulation of the key effectors in cell death execution pathway. HMM treatment was at 75 µg/mL (IC50+35 µg/mL) in ORL-204 for 24, 48, and 72 h. This concentration was selected for dosing to better capture caspases' activity that spans from 24 h onwards. At 24 h, HMM increased caspase-8 and -9 activities significantly in the treated cells (Fig. 2a). It is predicted that the active caspase-8 and -9 had subsequently activated the downstream executioner caspase as demonstrated in Fig. 2b where caspase-3/7 activity was increased up to 2-fold over a period of 72 h in HMM-treated cells as compared to the untreated control. This suggests that HMM induced apoptosis in a caspase-dependent manner via both the extrinsic and intrinsic signaling pathways. The cleavages of caspase-3/7 will presumably lead to the activation of endonuclease and protease as well as a series of cytomorphological changes including chromatin condensation and nuclear fragmentation (30). This is supported by higher numbers of apoptotic bodies (manifested as cell morphology alterations in the form of shrunken and fragmented cells) in ORL-204 treated with HMM at 40 µg/mL (IC50) for 72 h (Fig. 3).

Apoptosis is often linked to proliferation as they share the same set of regulators such as c-Myc, p53, pRb, Ras, PKA, PKC, Bcl-2, NF-κB, CDK, cyclins and CKI (31). This knowledge prompted us to look into the cell cycle profile of ORL-204. Cells were treated with HMM at IC50 and IC75 (250 µg/mL) for 72 h prior to staining and data acquisition via FACS analysis. Cell death is prominent in the group of cells treated with HMM at IC75; with accumulation of cells in the sub-G0/G1 peak which may be indicative of DNA fragmentation due to apoptosis (Table 2). Further to that, our earlier demonstration of upregulated caspase-3 activities in HMM-treated cells is indicative that HMM might have mediated the cleavage and inactivation of p21 that converts cancer cells from growth arrest to apoptosis (32). HMM arrested ORL-204 at G0/G1-phase in the cell cycle where there was a minor but statistically significant increment on the cell population in the G0/G1-phase and subsequently a decreasing trend in the G2/M-phase (Table 2). OSCC cell lines have been shown to overexpress cdk4 and cdk6; the key players in G1 phase (33),
thus suggesting that these cell lines are more sensitive to G1 inhibitor. HMM might have acted as a cdk inhibitor that impedes downstream functions.

A previous work done using wild type *L. rhinocerus* revealed a novel water-soluble polysaccharide-protein complex that could potentially be immunomodulatory agent for cancer immunotherapy (34) and Sum *et al.* (28) have reported that *L. rhinocerus* TM02® regulated the release of several cytokines/chemokines which are associated with tumorigenesis by RAW 264.7 murine macrophages, in particular the MIP2 and TIMP1. We proceeded to question if TM02® also demonstrated comparable immunomodulating properties in oral cancer in addition to its selective anti-proliferative property. We investigated the regulation of these cytokines release in ORL-204 over a period of 72 h post-HMM treatment at 10 µg/mL (IC_{25}) and 40 µg/mL (IC_{50}). HMM significantly inhibited the release of MIP2 from ORL-204 by 30 to 80 % in a dose-dependent manner while no effect was observed for TIMP1 (Fig. 4a). In most cancers, MIP2 expression is up-regulated for cell proliferation promotion and metastasis (35). Its secretion inhibition by HMM therefore suggests a repressive effect of HMM towards ORL-204 growth while its associative role in the alteration of osteoclastic activity remains unknown. It has been reported that TNF-α mediated the increase of MIP2 mRNA via NF-κB/MAPK, caspase-3 signaling pathway in macrophages (36) but the question as to whether similar mechanisms are applicable to our current study remains to be determined. However, in view of its anticancer effect by caspase-3/7 activities promotion and MIP2 secretion suppression, it is suggestive that HMM possesses antagonizing and/or multiple involvements in the TNF signaling pathway.

Many of the cytokines and mediators of inflammatory pathways are involved in the different steps of tumorigenesis (37). Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to prevent cancer and stop tumor growth by inhibiting prostaglandin synthesis through COX-2 hindrance (38). We further investigated the potential role of COX in regulating the inhibitory effect of HMM on ORL-204. Cells were treated with HMM at IC_{50} and the COX levels were determined (Fig. 4b). The selective inhibition of HMM towards COX-2 might indicate its potential role against development of oral lesions by inhibiting and/or interrupting the oral carcinogenesis pathway, further strengthening the therapeutic potential of COX-2 inhibitors in oral cancer treatment (39). There has also been increasing evidence that COX-2 produced prostaglandins that intervene tumor cell proliferation while some selective COX-2 inhibitors such as nabumetone inhibit proliferation of various COX-2-expressing cancer cells by a G0/G1-phase cell cycle arrest (40). Our current findings point to a possible linkage between COX-2 inhibitory effect and
G0/G1-phase arrest in ORL-204. However, due to the limitations of an in vitro study, the association remains to be elucidated.

From this study, we showed that HMM induced apoptosis in ORL-204 cells via the activation of caspase-3/7 through the extrinsic and intrinsic signaling pathways. HMM further manipulated cell cycle by arresting the cells at G0/G1-phase. Several molecules such as MIP2 and COX-2 related to TNF signaling contributed to the anticancer effect of HMM towards ORL-204 where both immunomodulators have been implicated in cell proliferation and inflammation (Fig. 5). A halted COX-2 expression has been shown to decrease MIP2 (41). Lee et al. (42) also reported that HMM attenuated TNF-α activity in LPS-induced RAW 264.7 cells, signifying an anti-inflammatory/immunosuppressive effect. HMM contains abundant amounts of carbohydrates and proteins (4 % by dry mass (m/m)) (42), suggesting the existence of polysaccharide-protein complexes in the fraction. The conglomeration of molecules in HMM may has contributed to its diverse roles in targeting various signaling pathways such as apoptosis, inflammation, and immunomodulation in order to exert its anticancer effect.

CONCLUSIONS

This is the first study to reveal the capability of L. rhinocerus TM02® in aiding oral cancer treatment and/or as a form of preventive measure against tumorigenesis by the intervention of the TNF signaling. The action of biting/chewing the sclerotium which have been practiced traditionally may now have an insightful implication. CWE demonstrated selective cytotoxic effect to ORL-48 and ORL-204 cell lines with little cytotoxicity towards primary human fibroblast while HMM; a high-molecular mass polysaccharide-protein complexes from CWE, induced apoptosis and exhibited anti-proliferative activity against ORL-204 by G0/G1-phase cell cycle arrest and inhibition of several immunomodulators affiliated with the TNF signaling pathway such as MIP2 and COX-2. Further investigations including in vivo and downstream molecular works are warranted to strengthen the current findings. As a limitation of this study, future work will also incorporate anti-cancer drug(s) as positive control to test along with purified fractions.

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LiGNO Biotech Sdn. Bhd. provided the mushroom sample. Cancer Research Malaysia provided the ORL-48, -108 and -204 cell lines in this study.
FUNDING

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CONFLICT OF INTEREST

Szu-Ting Ng and Chon-Seng Tan are affiliated with LiGNO Biotech Sdn. Bhd., an industry that commercialises tiger milk mushroom. The authors declare they have no financial interests.

AUTHORS’ CONTRIBUTION

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hui-Yeng Yeannie Yap, Boon-Hong Kong, Chee-Sum Alvin Yap, Szu-Ting Ng and Chon-Seng Tan. The first draft of the manuscript was written by Hui Yeng Yeannie Yap and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Table 1. Cytotoxicity of TM02® against various human cell lines

<table>
<thead>
<tr>
<th>Human line</th>
<th>cell</th>
<th>ORL-48</th>
<th>ORL-188</th>
<th>ORL-204</th>
<th>Fibroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>CWE</td>
<td></td>
<td>230</td>
<td>360</td>
<td>310</td>
<td>&gt;500</td>
</tr>
<tr>
<td>HMM</td>
<td></td>
<td>115</td>
<td>135</td>
<td>40</td>
<td>&gt;250</td>
</tr>
<tr>
<td>MMM</td>
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<td>125</td>
<td>245</td>
<td>175</td>
<td>&gt;250</td>
</tr>
<tr>
<td>LMM</td>
<td></td>
<td>&gt;400</td>
<td>&gt;400</td>
<td>&gt;400</td>
<td>NP</td>
</tr>
</tbody>
</table>

IC$_{50}$ value was determined from a mean plot of cell viability percentages against concentrations curve (N≥2)
Table 2. Effect of HMM on ORL-204 cell cycle distribution

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G0/G1-phase (%)</th>
<th>S-phase (%)</th>
<th>G2/M-phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>43.84±1.22</td>
<td>11.22±2.51</td>
<td>37.82±1.26</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>47.51±2.30*</td>
<td>12.41±3.40</td>
<td>23.10±3.24*</td>
</tr>
<tr>
<td>IC$_{75}$</td>
<td>49.10±1.67*</td>
<td>13.60±2.91</td>
<td>17.93±1.94*</td>
</tr>
</tbody>
</table>

Cells were treated with HMM for 72 h at 40 µg/mL (IC$_{50}$) and 250 µg/mL (IC$_{75}$). Cell distribution (%) in G0/G1-phase, S-phase and G2/M-phase are mean±SD (N=3; * p<0.05)

Fig. 1. Cytotoxicity of TM02® sclerotial cold water extracts upon 72 h treatment in ORL-48, -188 and -204 cells. Viability of cells was determined by calculating the relative cell viability in MTT assay. Values are as mean±SD. Abbreviation: CWE, TM02® sclerotial cold water extract
Fig. 2. Upregulation of: a) caspase-8, -9 and b) -3/7 activities in ORL-204 cells upon high molecular mass fraction (HMM) treatment over a period of 72 h at 75 μg/mL. Data were expressed as fold change in relative to the untreated control which was set as 1 (mean±SD, N=2; * p<0.05)
Fig. 3. Cell morphology alterations of ORL-204 cells: a) control, b) treated with high molecular mass fraction at 40 μg/mL for 72 h
Fig. 4. Regulation of modulators in HMM-treated ORL-204 cells: a) Regulation of cytokines (MIP2 and TIMP1) release by HMM in ORL-204 cells. Cells were treated with HMM for 72 h at 10 µg/mL (IC_{25}) and 40 µg/mL (IC_{50}), and b) effects of HMM on cyclooxygenases COX-1 and COX-2 activity in ORL-204 cells. Cells were treated with HMM for 72 h at 40 µg/mL (IC_{50}). Values expressed as mean±SD (N=3; * p<0.05)
Fig. 5. Proposed TNF cell signaling pathway intervention by high molecular mass fraction (HMM) at different phases. Anticancer effects of HMM in ORL-204 via apoptosis, proliferation inhibition (cell cycle arrest), immunomodulation and anti-inflammation (41) by means of targeting multiple cell signaling pathways and transcription factors. TNFR1, Tumor necrosis factor receptor 1; TRADD, TNFR1-associated death domain; FADD, Fas associated via death domain; BID, BH3 interacting-domain death agonist; Cyt c, Cytochrome complex; PGs, Prostaglandins; CXCL2, Chemokine (C-X-C motif) ligand 2 (MIP2 equivalent); CXCR2, CXC chemokine receptor 2