ORIGINAL ARTICLE

Effects of *Apis dorsata* Honey on the mRNA Expression of Selected *CYP450*, Pro-apoptotic, and Anti-apoptotic Genes during Induced Cytotoxicity in Cyclophosphamide-treated Human Lung Carcinoma (A549) Cells

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ABSTRACT

Introduction. One of the novel strategies in cancer treatment is the combination of conventional chemotherapeutic drugs and natural products. In a previous study, co-treatment of the anti-cancer drug cyclophosphamide (CP) with honey from giant honey bee (*Apis dorsata*) resulted to a dose-dependent increase in its cytotoxic effect in human lung carcinoma (A549) cells. However, the molecular mechanism of this combinatorial effect remains unknown.

Objectives. In this study, the effect of *A. dorsata* honey on the expression of selected CYP450 genes at the mRNA level, as well as the proapoptotic gene *CASP8* and antiapoptotic gene *BCL2* was investigated in CP-treated A549 cells.

Methods. MTT Assay was performed to determine the cell viability of A549 cells after treatment with CP with or without *A. dorsata* honey, as well as the EC_{50} of CP with honey thereafter. RT-qPCR was then performed to study the effect of *A. dorsata* honey on the expression of selected CYP450 genes as well as *CASP8* and *BCL2* genes in CP-treated A549 cells. LC-MS was carried out to screen for putative compounds in *A. dorsata* honey which may possibly have anti-cancer activity.

Results. Honey in the lowest concentration (0.6% v/v) most effectively enhanced the cytotoxic effect of CP. *CYP2J2* and *CYP1B1* indicated a 2.38-fold and 1.49-fold upregulation, respectively as compared to untreated cells. This cytotoxic effect is further enhanced by upregulation of *CASP8* that is paralleled by a downregulation of *BCL2*. Phytosphingosine and sphinganine are honey constituents which may be linked to the increased cytotoxicity of CP observed in A549 cells.

Conclusion. This study provides further knowledge on the molecular basis by which *A. dorsata* honey potentiates the cytotoxic effect of cyclophosphamide in A549 cells.

Keywords: Apis dorsata honey, human lung carcinoma (A549) cells, cyclophosphamide, MTT assay, RT-qPCR, LC-MS, apoptosis

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INTRODUCTION

Cancer is unarguably one of the top causes of morbidity and mortality in the world. At present, one of the treatment options for cancer involved the use of various chemotherapeutic drugs. Cyclophosphamide (CP) is one of the most successful antineoplastic agents that is widely utilized even today. It specifically targets cancer cells as an alkylating agent, causing damage to their DNA via the formation of intra- and inter-strand crosslinks.[1] However, the downside of the use of cyclophosphamide is that at cumulative doses, it may cause unwanted toxic effects such as bone marrow suppression, hemorrhagic cystitis, and carcinogenesis, threatening healthy non-cancer cells.[2]

In the recent years, there has been a growing interest into natural products which can be used as an adjunct to chemotherapeutic drugs to reduce their adverse toxic effects to normal cells.[3] Honey is a natural substance comprised of various bioactive compounds that is reported to show an increased and specific cytotoxic effect to cancer cells in combination with some anticancer drugs.[4,5] In 2013, Fernandez-Cabezudo et al.[5] worked on a method to suppress the toxicity of the chemotherapeutic drug, paclitaxel, in combination with manuka honey. Manuka honey is derived from nectar collected by Western honey bees (*Apis mellifera*). In this study, the antiproliferative activity of manuka honey on three different cancer cell lines in vitro were investigated in vitro as well as on male mice as animal models. It was shown that co-treatment of the chemotherapeutic drug with manuka honey leads to an effective cytotoxicity to cancer cells while decreasing nonspecific drug toxicity. This is associated with a caspase 9-dependent apoptotic pathway activation, leading to the induction of caspase-3, reduced BCL-2 expression, DNA fragmentation, and cell death.

Interestingly, honey from *Apis dorsata*, an endemic honey bee species to Asia, was shown to enhance the cytotoxic effect of cyclophosphamide in human lung carcinoma (A549) cells with a reduced cytotoxicity when compared with normal murine fibroblast cells.[6] Although it has been established that *A. dorsata* honey potentiates the anti-cancer effect of cyclophosphamide, the mechanism by which honey enhances cytotoxicity in cancer cells are yet to be elucidated.

Cytochrome P450s (CYPs) are membrane-associated proteins involved in the metabolic activation and inactivation of multiple endogenous and exogenous compounds.[7] CYPs are believed to be associated with the metabolic activation of inactive prodrugs such as cyclophosphamide. Hydroxylation on the oxazaphosphorine ring by hepatic cytochrome P450 enzymes is a key step in the chemical activation of cyclophosphamide to release phosphoramide mustard which is responsible for its cytotoxic effects.[1] Due to their xenobiotic-metabolizing ability, CYPs are considered to play a vital role in cancer susceptibility. In fact, the differential expression of CYP isoforms in cancer cells is seen as novel approach in tumor-specific cancer treatment.[8] Tumor-specific prodrug bioactivation spares normal cells from the toxic side effects of chemotherapeutic drugs. In A549 cells, the differential expression of particular CYP450 genes particularly *CYP2J2* and *CYP1B1* have been documented by several authors.[9,10]

This study aimed to determine the effects of *A. dorsata* honey on the expression of selected CYP450 genes in CP-treated A549 cells via reverse transcription - polymerase chain reaction (RT-PCR). The findings in this present study provides further knowledge on the molecular basis by which *A. dorsata* honey potentiates the cytotoxic effect of cyclophosphamide in A549 cells.

MATERIALS AND METHODS

Materials and Reagents

The basal media used for cell culture was RPMI1640, procured from Medical Test Systems Inc. Fetal bovine serum (FBS), penicillin-streptomycin (PNS), phosphate buffered saline (PBS), and trypsin were provided by Globetek Science Foundation, Inc.

The MTT Cell Proliferation and Cytotoxicity Assay Kit containing MTT dye and formazan diluent was procured from Biorbyt. Cyclophosphamide with brand name Cyphos was obtained from the Philippine General Hospital (PGH) Oncology Pharmacy in 500 mg vials. The honey sample was procured from a distributor of virgin honey from the native *A. dorsata* bee species of Palawan, Philippines. The *A. dorsata* honey was reconstituted in distilled water, and filter-sterilized using a 0.04 μm syringe filter.

M-MuLV first strand synthesis components were provided by Globetek Science Foundation, Inc. while SensiFAST™ SYBR No-ROX kit was obtained from BioTriune Inc. Primers were ordered from Macrogen. The phenol chloroform isoamyl reagent, nuclease-free water, and qPCR tubes were obtained from Globetek Science Foundation, Inc. as well.

The materials and chemicals employed for the mass spectrometry were courtesy of the Department of Biochemistry and Molecular Biology, University of the Philippines - College of Medicine.

Cell Line, Culture, and Treatment

The human lung carcinoma (A549) cells were generously provided by Globetek Science Foundation Inc. A549 cells were cultured in RPMI1640 with 10% Fetal Bovine Serum (FBS) and incubated at 37°C in a 95% air/5% CO_2 atmosphere at constant humidity. The A549 cells were cultured in a T75 culture flask until they reached ~80% confluency. Cells were then harvested by washing them first with 5 mL phosphate buffered solution (PBS) and adding 5 mL 0.025% trypsin. The cells were centrifuged at 500 rcf for 5 min and resuspended in growth media. The resuspended cells were counted using an automated cell counter (Invitrogen) and seeded in a 96-well microtiter plate

at a density of 1 × 104 cells/100 µL–1 well–1. The cells were allowed to adhere for 24 h at 37°C in a CO_2 incubator.

A549 cells were treated according to the treatment design of Dalet et al.[6] Six groups of 18 wells each was assigned, corresponding to different concentrations of cyclophosphamide (CP) treatment (0, 1.25, 3.75, 6.25 and 8.75 mg/mL). Each group was further subdivided into 6 groups of 3 wells each, corresponding to 1 group without honey, 4 groups treated with varying concentrations of *A. dorsata* honey (0.6%, 1.25%, 2.5% and 10% v/v), and an untreated (negative) control group which contains cells alone. In addition, a blank control group in which no cells were seeded was also assigned. All treatment and control groups were done in triplicates and was left for 24 h at 37°C in a CO_2 incubator.

Experimental Design

This study involved three phases namely: MTT Assay followed by Gene Expression Analysis and then by Mass Spectrometry. Phase 1 was performed to determine the cell viability of A549 cells after treatment with cyclophosphamide (CP) with or without *A. dorsata* honey and to determine the EC_{50} or the half maximal effective concentration of CP with honey thereafter. Meanwhile, Phase 2 was performed to study the effect of *A. dorsata* honey on the expression of selected CYP450 genes as well as pro- (*CASP8*) and anti-apoptotic (*BCL2*) genes at the mRNA level in cyclophosphamide-treated A549 cells by quantitative reverse-transcriptase-polymerase chain reaction (RT-qPCR). Phase 3 was carried out to screen for putative compounds in *A. dorsata* honey which may possibly have anti-cancer activity.

MTT Assay

After exposure to the different concentrations of CP and CP+honey treatments, ten (10) µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye was applied to all the wells of each group. The formation of formazan crystals by the A549 cells were viewed under an inverted microscope. After 4 h, 100 µL of formazan diluent was also added to all the wells of each group. The absorbance measurements were measured using a microplate reader at 570 nm. Consequently, the absorbance data obtained were used to create a dose-response curve and calculate the half maximal effective concentration (EC_{50}) value of the CP and CP+honey treatments using licensed GraphPad Prism 5.0. The EC_{50} value of the most effective CP+honey treatment obtained from Phase I was used in the treatment of A549 cells to CP and CP+honey for gene expression analysis.

Primer Design

The CYP450 genes (*CYP2J2*, *CYP1B1*) selected for this study were based from earlier studies,[9–11] who reported on the differential expression of these CYP450 isoforms in the A549 cell line. Primers were designed using a combination of NCBI's Primer-BLAST, Ensemble, and Primer3Plus, and PrimerQuest based on the recommendation and supervision of Dr. Jay T. Dalet. A melting temperature range of 58°C-65°C was set during primer design with a melting temperature of 60°C given as the optimum values. The product size was limited to 200-300 bp and GC content between 45-55%. The presence of hairpin loops, self-dimers, and heterodimers was checked using PCR Primer Stats. The primer sequences are shown in Table 1 including the housekeeping genes used in the study.

Table 1. Primer Sequences Used in the Study

Gene	Primer Sequence (5'-3')
CYP2J2	F: TGGAGGTTCAGCTGTTTGTG R: ATTATGTCAAGTGGCCAGGC
CYP1B1	F: TCCTCCTCTCCAGGTAT R: GAGAACTTTGATCCAGCTCGA
CASP8	F: TAGGCAGGTTAGGGGACTCG R: GGTTCTTGCTTCCTTTGCGG
BCL2	F: TAGGCAGGTTAGGGGACTCG R: GGTTCTTGCTTCCTTTGCGG
GAPDH	F: GACTTCAACAGCGACACCCACTCC R: AGGTCCACCACCCTGTTGCTGTAG

Total RNA Extraction

The total RNA from the CP with *A. dorsata* honey-treated A549 cells was extracted using phenol chloroform isoamyl (PCI) as per protocol of the supplier (Globetek Science Foundation). Media from T75 flask was decanted and the A549 cells were scraped using a cell scraper. About 400 µL of the sample was transferred to 1.5 mL microcentrifuge tubes. An equal amount of TE buffer was added to the tubes. The cells were then mechanically lysed using a 3 mL syringe repeated fifteen (15) times. Then, 10 µL of DNAse I was added to the tubes and were incubated for 5 min in ice. Next, about 10 µL of proteinase K was added to the tubes and were incubated for 10 min. After which, an equal amount of phenol chloroform isoamyl was added to the tubes. The tubes were then centrifuged at 10,000 × g for 10 min. The interphase and aqueous layer were collected and placed in separate microcentrifuge tubes. The samples were then precipitated using absolute ethanol and spun at 13,000 × g for 5 min. Then, the samples were washed with 75% ethanol and were spun again at 13,000 × g for 5 min. The micro-centrifuge tubes were left to stand and air-dry for about 10 min. Lastly, the pellet was resuspended in TE buffer by pipetting up and down for several times.

RNA quantitation

The total RNA extracted was quantitated using Qubit™ (Invitrogen) assays according to the protocol recommended by the manufacturer. A working solution consisting of 1 µL Qubit™ reagent diluted in 199 µL Qubit™ buffer was prepared in a clean plastic tube. Standard solutions were also made by mixing 190 µL of the working solution with

10 µL of the appropriate standard stock solution. Then, a volume of 1 µL RNA sample was added to a 0.5 mL PCR tube containing 199 µL of the working solution resulting to a final volume of 200 µL. The PCR tube was then vortexed for 2-3 seconds and incubated for 2 min at room temperature. The PCR tube was inserted into the Qubit 2.0 Fluorometer and readings were taken.

cDNA Synthesis

Complementary DNA (cDNA) was synthesized from total RNA using M-MuLV first strand synthesis components (New England Biolabs Inc.) following the protocol of Globetek Science Foundation. The reaction mix was prepared in a 0.2 mL PCR tube by mixing 5 µL of total RNA, 2 µL 10X M-MuLV standard buffer, 1 µL M-MuLV reverse transcriptase, 2 µL oligo-dT primer (50 µM), and 10 µL nuclease-free H2O with a total volume of 20 µL. A negative (no RT) control was also prepared using the same components except M-MuLV reverse transcriptase. After which, the samples were loaded in the thermal cycler: 25°C for 5 min annealing, 42°C for 60 min reverse transcription, 65°C for 20 min enzyme inactivation, and 4°C chilling. The reaction mix was then stored at -20°C.

Quantitative Polymerase Chain Reaction (qPCR)

Real-time or qPCR (Bioline) was done following the manufacturer's protocol. The reaction mix consisted of 5 µL of 2X SensiFAST™ SYBR No-ROX mix, 0.5 µL of 10 µM forward primer and 0.5 µL of 10 µM reverse primer (for each gene), 2.5 µL of cDNA template and nuclease-free water as required. The final volume was 10 µL per reaction. The qPCR tubes were then loaded in the real-time thermocycler (Bio-Rad CFX96) and three-step cycling was performed. The thermocycler was set as follows: one cycle for polymerase activation at 95°C for 2 min, 40 cycles of PCR (95°C, 5 s; 58°C, 10 s; 72°C, 20 s). The gene expression profiles of *CYP1B1*, *CYP2J2*, *CASP8*, and *BLC2* at the mRNA level were then quantified by calculating their fold changes in treated and untreated A549 cells. Meanwhile, GAPDH was used as loading dose.

Mass Spectrometry

Prior to the LC-MS procedure, *A. dorsata* honey samples were first dissolved in methanol. Forty (40) µL of the sample was dissolved in 1 mL of 3:1 methanol:water (1:50 dilution). After which, the solution was then passed through a 0.2 µm PTFE syringe filter prior to LCMS injection. A 2.1 × 100 mm 1.8-micron C18 column was used to separate the metabolites. The mobile phase A was water+0.1% formic acid while mobile phase B was acetonitrile+0.1% formic acid. The column temperature was at 40°C. The injected volume was 1 µL. The flow rate was maintained at 0.5 mL/min. Leucine enkephalin was used as a reference for mass correction. (Table 2)

Table 2. Mass Spectrometry Results

Time (min)	Flow rate (mL/min)	Composition A (%)	Composition B (%)	Curve
0.00	0.500	95.0	5.0	Initial
0.50	0.500	95.0	5.0	6
15.00	0.500	5.0	65.0	6

For the MS portion of Phase 3, the following parameters were employed: capillary voltage: 1.0 kV (EsI+), source temperature: 120°C, desolvation temperature: 550°C, cone voltage: 40 V, cone gas flow: 40 L/h, desolvation gas flow: 950 L/h, scan range: 50-1200 m/z. This portion was done in three runs.

Accurate mass screening was carried out through the Waters UNIFI Scientific Information System v1.8.1.073. The mass peaks and m/z ratios were calculated by the Sum Peak algorithm. Only the high intensity peaks were used to find their m/z ratios. The base peak ions of distinct peaks were subjected to library matching using the Traditional Chinese Medicine (TCM) library and ChemSpider. Annotation of the candidate masses was based on the accurate mass match, isotopic ratio match, and precursor ion intensity counts. The m/z ratios were then used for the identification of the metabolite through the METLIN database. A criterion of 15 ppm mass accuracy was required.

Statistical Analysis

The results were presented as mean ± SEM. Unpaired Student's distribution test (t-test) was used to determine significant difference between the groups. A value of $p < 0.05$ was considered a significant difference between the groups.

RESULTS

Phase I: MTT Assay

The effects of varying concentrations of *A. dorsata* honey on the cytotoxicity induced by cyclophosphamide (CP) in treated A549 cells are shown in Figure 1. Treatment with honey (without CP) lead to significant increase in cytotoxicity that reached up to 25% at the highest honey concentration. It can be observed that *A. dorsata* honey (ADH) was most effective on promoting cytotoxicity at the lowest concentration of 0.6% (v/v), with a maximum increase of up to 4.3% cytotoxicity in combination with 3.75 mg/mL CP ($p < 0.05$). However, it is noteworthy that a concentration of 1.25% ADH also consistently lead to a significant increase in cytotoxicity, leading to a maximum of 5.2% increase in cytotoxicity as compared to untreated control cells ($p < 0.05$). Generally, honey in low concentrations (0.6% and 1.25%) increased the cytotoxicity of CP. Meanwhile, honey in higher concentrations (2.5% and 5%) lead to a significant decrease in cytotoxicity in combination with 1.25 mg/mL and 6.25 mg/mL CP, respectively. The highest magnitude of decrease in cytotoxicity was observed in the combination of 5% ADH and 6.25 mg/mL CP, leading to a 4.6% decrease in cytotoxicity ($p < 0.05$).

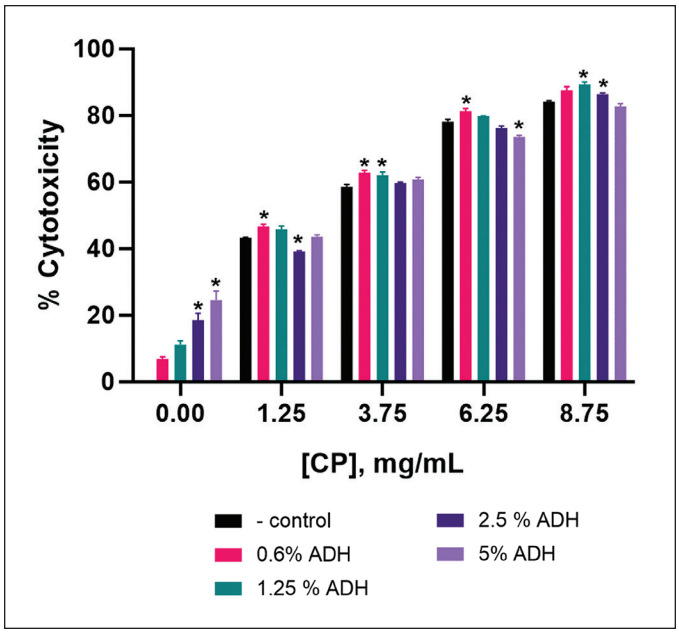

Figure 1. Mean cytotoxicity SEM of cyclophosphamide (CP)-treated A549 cells under the influence of various doses (%v/v) of *A. dorsata honey* (ADH) in A549 cells. The ability of the treated cells to reduce MTT to formazan was estimated and expressed as a percentage of untreated control cells.

*(Significant difference against untreated control cells, *p value <0.05). ADH = A. dorsata honey.*

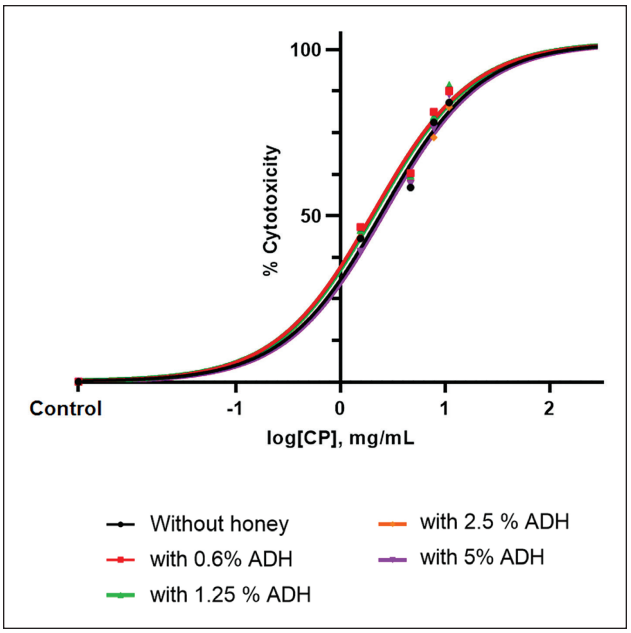

Figure 2. Dose-response curves of CP and CP+honey treatments in A549 cells. Untreated A549 cells represent the control group.

ADH = A. dorsata honey.

The dose-response curve (Figure 2) of the CP+honey treatments on A549 cells revealed a leftward shift from that of the negative control (CP without honey). This leftward shift is correlated with a decrease in EC_{50} values as seen in Table 3, with the highest in magnitude at a honey concentration of 0.6% (v/v). At this honey concentration, the EC_{50} shifted from 5.677 (negative control) to 4.364. Treatment with ADH generally led to a leftward shift in the dose-response curve of CP except for the highest honey concentration of 5% (v/v) in which a slight rightward shift from the negative control was observed. This is associated with an increase in EC_{50} value from 5.677 to 5.746.

Phase II: Gene Expression Analysis

The CP+ honey combination which resulted to the greatest cytotoxic effect was used for the gene expression analysis. Using the EC_{50} values obtained in Phase 1, A549 cells were exposed to CP and CP+honey for 24 h. RT-qPCR was then performed and the fold changes obtained from qPCR was used to quantify the gene expressions of the target genes at the mRNA level in the treatment group relative to the negative control (honey untreated cells) and a housekeeping gene (GAPDH).

The gene expression profiles of *CYP2J2*, *CYP1B1*, pro-(*CASP8*) and anti-apoptotic (*BCL2*) genes in *A. dorsata*

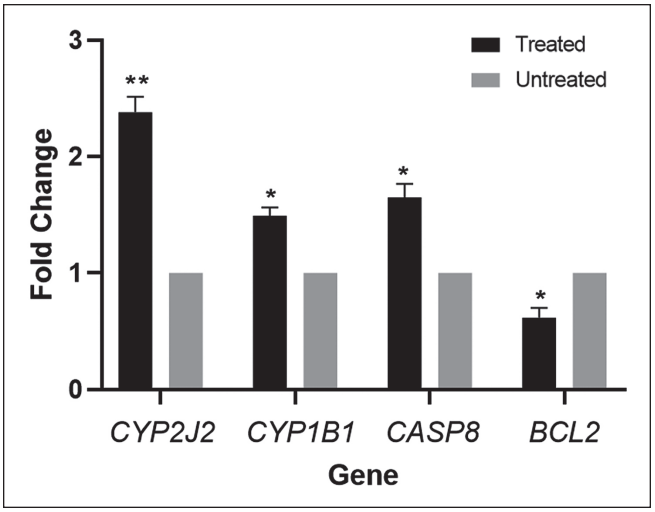

Figure 3. Relative expression of selected *CYP450* (*CYP2J2* and *CYP1B1*), proapoptotic (*CASP8*), and anti-apoptotic (*BCL2*) genes expressed in terms of fold changes in qPCR. A default value of 1 was assigned to each untreated control.

*(Significant difference against honey untreated control cells, *p value <0.05, **p value <0.01).*

Table 3. EC_{50} Values Generated from the Dose-response Curves of CP and CP + Honey Treatments

Treatment	EC_{50} in A549 cells (mg/mL)
CP	5.677
CP+0.6% ADH	4.364
CP+1.25% ADH	4.403
CP+2.5% ADH	5.644
CP+5% ADH	5.746

Table 4. Putative Compounds from *A. dorsata* Honey as Identified from Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

m/z	Retention time (min)	Putative compound	ppm	Possible activity	Reference
261.1212	0.5768	Heterodendrin	4	Induces cyaninde toxicity in neoplasms	12
317.2930	7.8959	Lotaustralin	5	Induces cell death in human T-cell lymphoma	13
301.2981	9.0284	Phytosphigosine	8	Inhibits proliferation and induces apoptosis in human colon cancer cells	14

and CP-treated A549 cells are summarized in Figure 3. Treatment with *A. dorsata* honey resulted to an upregulation of *CYP2J2* genes indicated by a significant 2.38-fold change in comparison to untreated cells.

Similarly, *CYP1B1* was upregulated in CP + honey-treated A549 cells (Figure 2). The treated cells achieved a 1.49-fold change in the expression of *CYP1B1* genes compared to untreated cells.

The CP+honey treatment of A549 cells resulted to an upregulation of the proapoptotic gene *CASP8* and a downregulation of the anti-apoptotic gene *BCL2*. For *CYP2J2*, a 1.65-fold change in was observed in treated cells relative to untreated cells. For *BCL2*, however, the fold change for the treated cells was relatively lower as compared to that of untreated cells, which means that *BCL2* was down-regulated. It must be noted that mRNA expression may not correlate with protein expression.

Phase III: Mass Spectrometry

The base peak ion (BPI) chromatogram produced from the LC-MS analysis of *A. dorsata* honey is shown in Figure 4. Peak annotations in the chromatogram are the retention time of the most intense peak in each spectrum which correspond to a particular putative substance present in *A. dorsata* honey. Annotation of the candidate masses was based on the accurate mass match, isotopic ratio match,

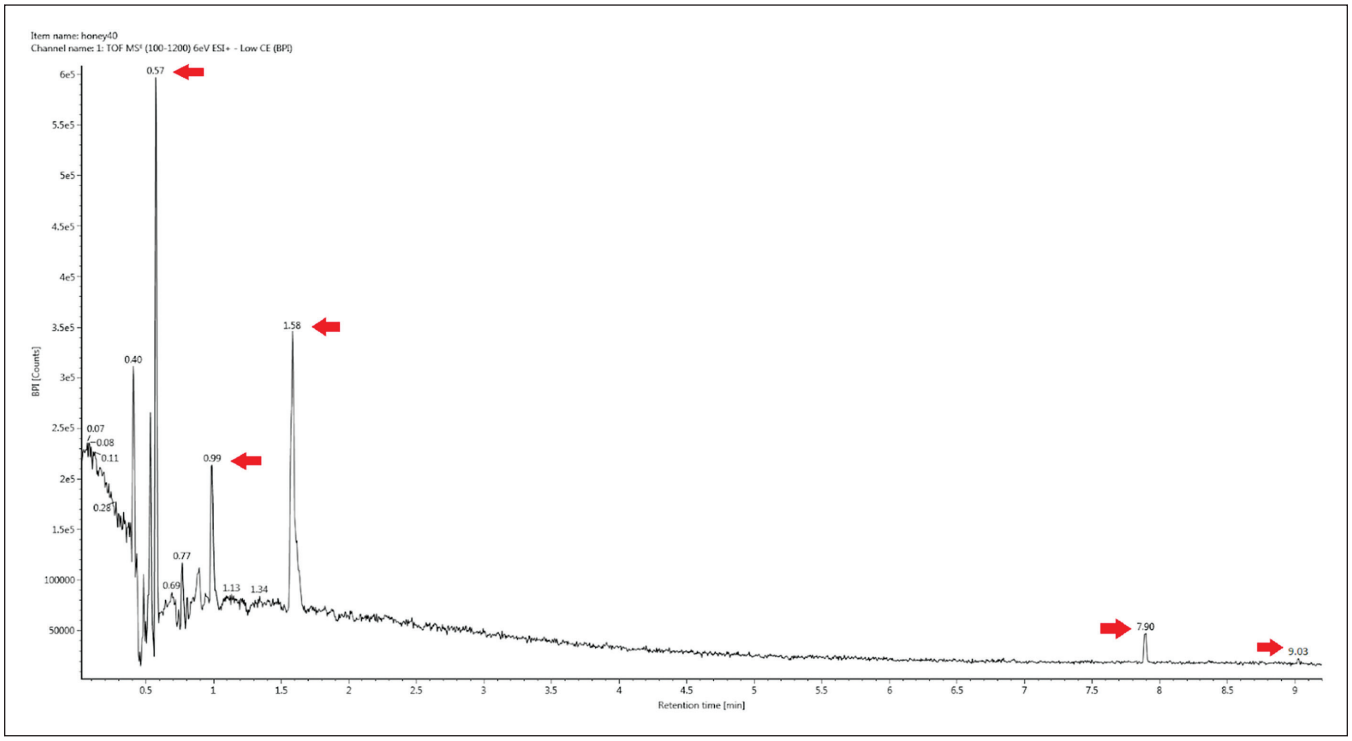

Figure 4. Base peak ion (BPI) chromatogram of *A. dorsata* honey dissolved in methanol. The figure shows the peak pattern of non-targeted substances from *A. dorsata* honey. The peaks are illustrative m/z-values as obtained from the corresponding MS spectrum. Red arrows indicate the distinct peaks from each respective retention time, which were used in the identification of putative compounds present in the *A. dorsata* honey sample as shown in Table 4. (LC-MS run time: 15 min; injected volume: 1 µL; flow rate: 0.5 mL/min).

and precursor ion intensity counts. Of these items, only the five most distinct peaks were selected for identification using the METLIN database.

The results of the match search using METLIN database is summarized in Table 4. Out of the five distinct peaks shown in Figure 4, only four compounds were selected as putative substances present in *A. dorsata* honey that possess possible anticancer activity (see Appendix for the specific fragmentation patterns). Apparently, the putative compounds in the *A. dorsata* honey used in this study differ to that of Dalet et al.[6] which originated from Silang, Cavite. Only phytosphingosine, a sphingolipid analog, was identified in both *A. dorsata* honey samples. The putative compounds of *A. dorsata* honey identified in Table 4 can then be linked to its cytotoxic effect observed in Phase I.

DISCUSSION

Cancer remains to be one of the top causes of death worldwide despite the advances in the knowledge of cancer development and the increasing modalities in cancer treatment. One of the novel strategies in cancer treatment is the combination of conventional chemotherapeutic drugs and natural products. In this study, the combinatorial effect of the anti-cancer drug cyclophosphamide and honey from giant honey bee (*A. dorsata*) in the induction of cytotoxicity in human lung carcinoma (A549) cells was investigated.

Phase I: MTT Assay

The results of the cell viability assay confirm that *A. dorsata* honey, in low concentrations, enhances the cytotoxic effect of CP in treated A549 cells as previously established by Dalet et al.[6] For example, co-treatment of CP at the lowest honey concentration (0.6% v/v), significantly increased ($P<0.05$) the cytotoxicity induced by CP at CP doses 1.25, 3.75, and 6.25 mg/mL as compared to cells treated with CP alone (Figure 1). However, co-treatment of 1.25 mg/mL and 6.25 mg/mL CP with higher honey concentrations (2.5% and 5%) lead to a significant decrease in cytotoxicity as compared to CP-treated cells. Thus, co-treatment of honey with CP exhibits a biphasic effect that is dose-dependent as observed in the previous study.[6]

A dose-response curve was generated from the data set obtained from the MTT assay which was used to determine the EC_{50} values of CP and CP+honey treatments. In this study, EC_{50} is defined as the drug concentration resulting to 50% of the maximum response, that is, the % cytotoxicity induced by CP treatment. In A549 cells, a leftward shift from the dose-response curve of the negative control (honey untreated) cells was observed in all honey concentrations except for the highest honey concentration (Figure 2). This leftward shift is correlated with a decrease in the EC_{50} values of CP. For example, co-treatment of CP with the lowest honey concentration led to a decrease in the EC_{50} of CP from 5.677 to 4.364. This means that co-treatment of CP with 0.6% honey lowers the dose of CP required to elicit a cytotoxic effect to cancer cells. The biphasic effect of honey is also evident in the dose-response curve of CP. While a leftward shift was observed in CP treated with 0.6%, 1.25%, and 2.5% honey, a slight rightward shift was observed in the dose-response curve of CP upon treatment with 5% honey. This corresponds to an increase in the EC_{50} value of CP from 5.677 to 5.746. This means that co-treatment of CP with 5% honey reduces the potency of CP in inducing cytotoxicity in A549 cells. Phase I (MTT assay) can also be seen as a fine-tuning of the optimal dose of CP, in combination with *A. dorsata* honey, which can elicit half of the maximum response which is cytotoxicity in A549 cells. Since the lowest honey concentration (0.6% v/v) was most successful in eliciting this response in treated A549 cells, it was subsequently used in the gene expression analysis (Phase II).

It is noteworthy that the dose-response curve as well as the EC_{50} values generated in this study differs from that of Dalet et al.[6] despite using the same cell line. The EC_{50} values of CP and CP+honey treatments determined in this study were relatively lower than what was previously determined. Aside from the fact that a different range of *A. dorsata* honey concentrations were used in this study, this difference may also be attributed to the source of *A. dorsata* honey. The *A. dorsata* honey used in this study originates from Rizal, Palawan while in the previous study, the honey was obtained from Silang, Cavite. It must be noted that the specific properties of honey and its anticancer effects vary with factors such as the geographical region, climate, and flower source, as well its packaging and storage.[3] For this instance, the differences in the potency of *A. dorsata* honey in the inhibition of A549 cells may be attributed in part to the floral source and the environment to which the giant honey bee belongs.

Phase II: Gene Expression Analysis

Gene expression analysis of selected *CYP450*, *CASP8*, and *BCL2* genes was done to explain the possible molecular basis by which *A. dorsata* honey enhances the cytotoxic effect of CP in A549 cells. Gene expression here specifically pertains to the mRNA level. *CYP2J2* is believed to be involved in the bioactivation of CP extrahepatically[15] and the fact that it is differentially expressed in A549 cells[9] makes it an ideal target for this experiment. *CYP1B1* was also shown to be highly expressed in neoplasms including A549 cells[10] which also makes it a potential target for tumor-specific drug metabolism. However, previous studies revealed that *CYP1B1* is not involved in the bioactivation of CP.[16] Investigation of the gene expression profile of these two CYP isoforms that have different action towards CP can be useful in determining the extent to which *A. dorsata* honey can enhance the cytotoxic effects of CP in A549 cells. *CASP8* is a proapoptotic protein primarily involved in the extrinsic or death receptor-dependent apoptotic pathway whereas *BCL2* is a membrane-associated anti- apoptotic protein which inhibits the actions of pro-apoptotic proteins.

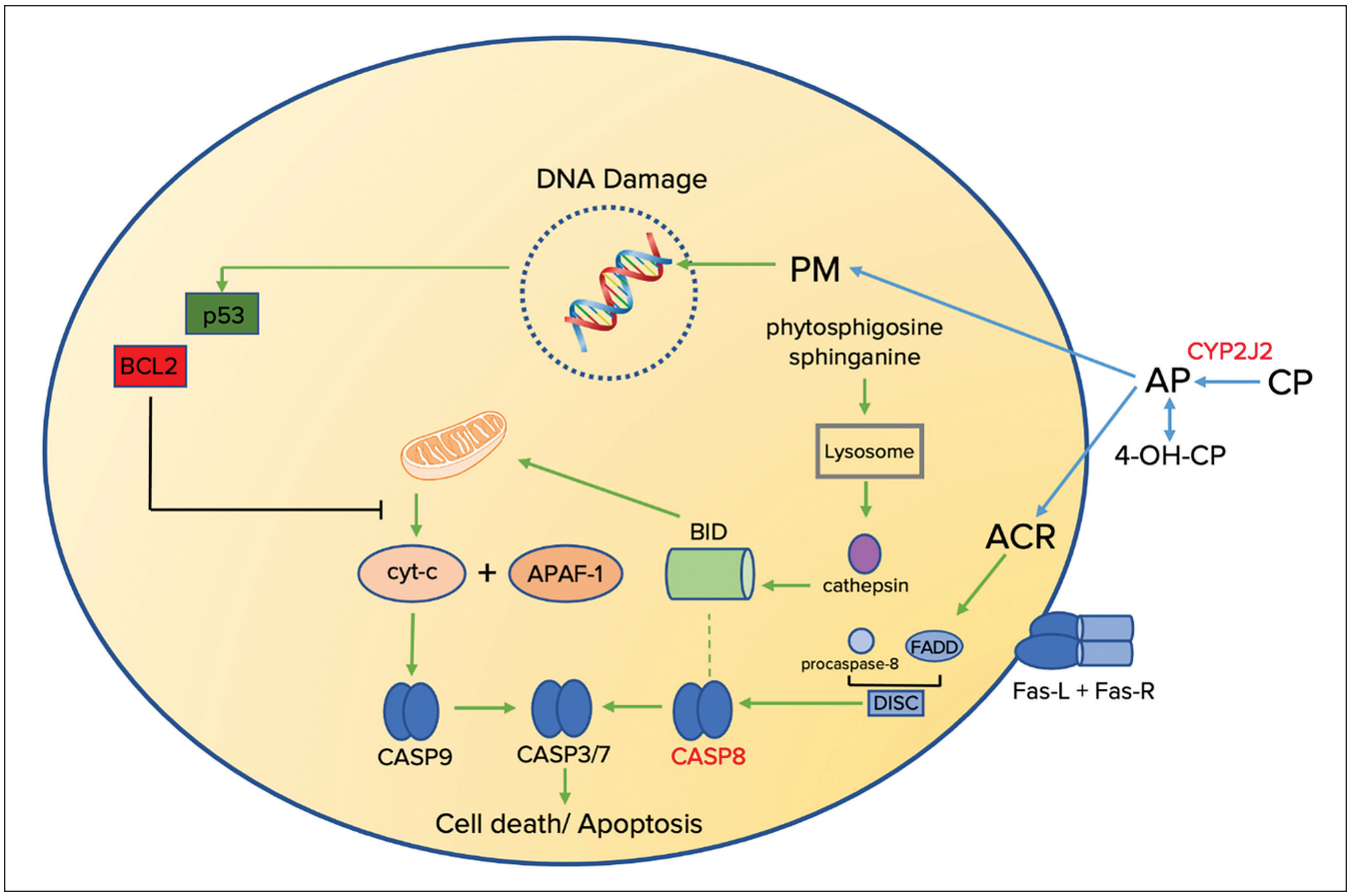

Figure 5. Proposed mechanism of CP + honey-induced cytotoxicity in A549 cells. Cyclophosphamide (CP) is bioactivated by *CYP2J2* giving rise to the intermediate metabolite aldophosphamide (AP), which is in equilibrium with its tautomer 4-hydroxycyclophosphamide (4-OH-CP). AP is converted to the toxic metabolites phosphoramide mustard (PM) and acrolein (ACR), both of which indirectly promotes apoptosis.

Modified from Ahmed and Othman (2013).[18]

The expression levels of these opposing genes can also show how *A. dorsata* enhances the cytotoxic effect of CP.

The results of the gene expression analysis of the *CYP450* (*CYP2J2* and *CYP1B1*), *CASP8*, and *BCL2* genes reveal a possible molecular mechanism that may explain the cytotoxic effect of CP-treated A549 cells with the influence of *A. dorsata* honey. Figure 5 is provided as reference for the following discussion.

A. dorsata honey promoted the expression of *CYP2J2* as indicated by a significant 2.38- fold upregulation in CP + honey-treated A549 cells relative to honey-untreated cells. The activation of CP to 4-hydroxycyclophosphamide is catalyzed by the hepatic CYP isozymes CYP2B6, -2C9 and -3A4 (with minor contributions from -2A6, -2C8 and -2C19).[17] In this study, *CYP2J2* enzymes may linked to the metabolic activation of CP in A549 cells.

The differential expression of *CYP1B1* in extrahepatic neoplasms rather than in normal cells makes it a potential target for tumor-specific drug metabolism. While, tumor-specific drug metabolism by *CYP1B1* may represent a novel mechanism for cancer treatment, it is also involved in the inactivation of or resistance to some prodrugs.[19] Cyclophosphamide is prodrug that shows no inhibitory activity towards *CYP1B1* as shown in a study by Rochat et al.[16] The observed 1.49-fold upregulation of *CYP1B1* upon treatment with CP+honey reveals that despite the presence of the prodrug, *CYP1B1* still performs an endogenous function in A549 cells. The results from the gene expression analysis of the selected CYP450 isoforms, *CYP2J2* and *CYP1B1*, show that at a low concentration of *A. dorsata* honey, cytotoxicity in A549 cells is favored due to *CYP2J2* being relatively more upregulated than *CYP1B1*. However, it is possible that at a higher concentration of *A. dorsata* honey, *CYP1B1* would be more upregulated than *CYP2J2* and thus, *CYP2J2*-mediated bioactivation of CP would be suppressed by the endogenous function of *CYP1B1* that maintains the survival of the A549 cells. This is manifested by the dose-dependent potentiation of the cytotoxic effect of CP by *A. dorsata* honey as revealed in Phase I.

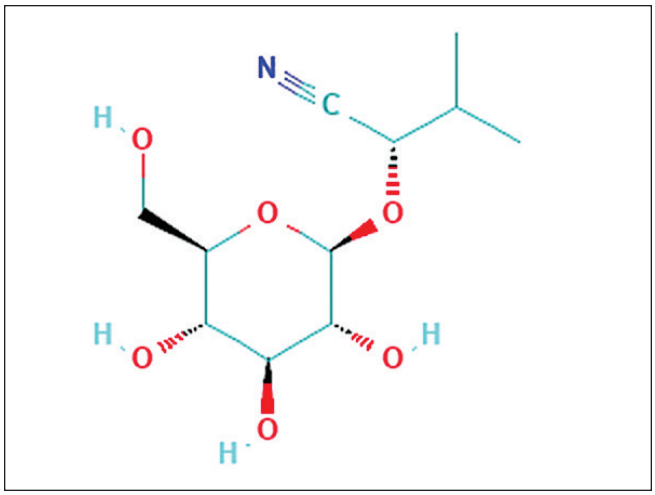

Figure 6. Chemical structure of heterodendrin.

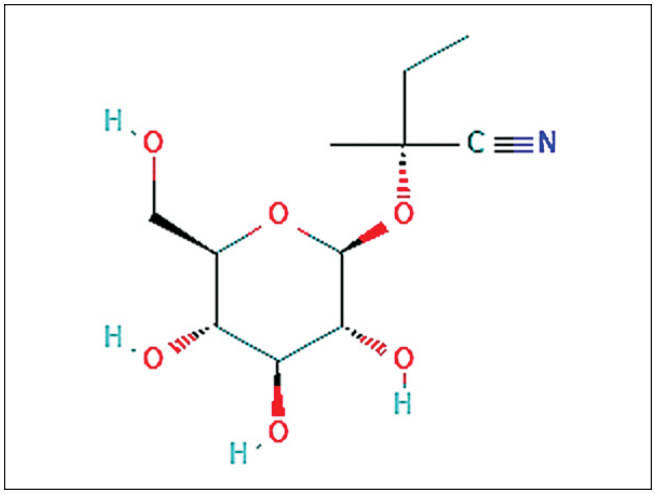

Figure 7. Chemical structure of lotaustralin.

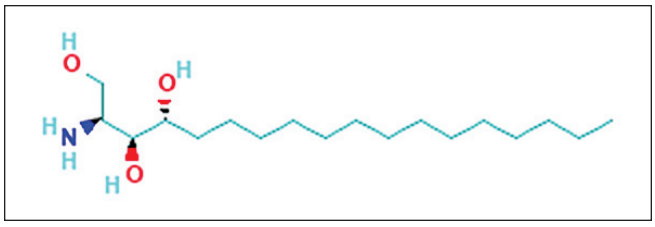

Figure 8. Chemical structure of phytosphingosine.

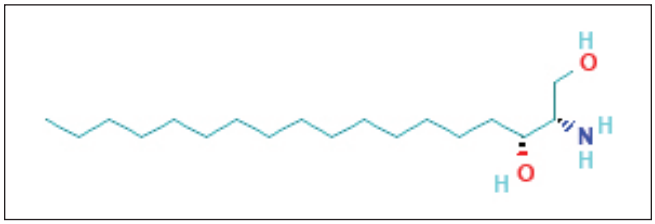

Figure 9. Chemical structure of sphinganine.

A. dorsata honey also promoted the expression of *CASP8* with a 1.65-fold upregulation in CP+honey treated cells compared to untreated cells. Several studies have already reported on the effect of honey and its constituents in the induction of apoptosis although the pathway involved is usually caspase-9-dependent. Fauzi et al.[4] reported that Tualang honey is cytotoxic and induces apoptosis in breast, ovarian, and HeLa cell lines by increasing the levels of caspase expression particularly the initiator caspase-9 and effector caspases-3/7. A similar pathway is exhibited by manuka honey, which was shown to induce late apoptotic events in murine melanoma, colorectal carcinoma, and human breast cancer cells.[5]

The increased expression of caspase-8 is another factor that may be attributed to the increase in the inhibition of A549 cells as observed in Phase I. In the extrinsic apoptotic pathway, apoptotic signals trigger the oligomerization of the Fas-associated death adaptor protein (FADD) as well as caspase-8, forming a death-inducing signaling complex (DISC) (Figure 5). Caspase-8 is autoactivated at the DISC which is followed by the activation of effector caspases that ultimately lead to cell death. The other CP metabolite acrolein, a highly reactive α,β-unsaturated aldehyde, also enhances this apoptotic pathway. In a study by Roy et al.[20] it was shown that p53 is an initiating factor in acrolein-induced death receptor activation during apoptosis in A549 cells. They showed evidence that acrolein triggers the death receptor pathway of apoptosis, as shown by the elevation of Fas ligand (Fas-L). Binding of the transmembrane Fas-L with its receptor results in the recruitment of the FADD. Acrolein also promotes the translocation of FADD to the plasma membrane which binds to procaspase-8 forming the DISC. With the caspase-8 being autoactivated at the DISC, downstream effectors of programmed cell death such as caspase-3, caspase-6, and caspase-7 execute cell death (Figure 5).[21]

CASP8 upregulation was also paralleled by *BCL2* downregulation which possibly promoted the execution of apoptosis in CP + honey-treated cells. *BCL2* is an anti-apoptotic gene that acts upstream of the caspase pathway that can block apoptosis in upregulated levels. *BCL2* is a membrane-associated pro-survival protein which plays an important role in inhibiting the actions of proapoptotic proteins such as caspases. One possible mechanism by which *BCL2* does this is by blocking cytochrome c release from mitochondria, which is important in the initiation of the caspase cascade (Figure 5).[21] The apoptotic pathway usually follows an increased expression of proapoptotic genes such as caspases and is associated with an inhibited expression of the antiapoptotic protein *BCL2*.[22] With a reduced expression of *BCL2* in CP + honey-treated cells compared to untreated cells, it can be said that the *BCL2* gene was downregulated with the influence of *A. dorsata* honey, thereby promoting the cascade of caspase-mediated apoptosis in A549 cells.

Phase III: Mass Spectrometry

The identification of putative compounds using LC- MS revealed four possible anti-cancer compounds present in the *A. dorsata* honey.

Cyanogenic glucosides are secondary plant metabolites that function in plant defense. These nitrogenous compounds liberate hydrogen cyanide upon hydrolysis in a process called cyanogenesis. Heterodendrin and lotaustralin (Figures 6 and 7) are examples of cyanogenic glycosides that are common among species of *Acacia* L. (Fabaceae: Mimosoidae).[23] According to Lecocq et al.,[24] cyanogenic glycosides are innocuous to honey bees as they were able to tolerate cyanogenic glucosides linamarin and amygdalin ingested from clover nectar at naturally occurring concentrations without any ill effects. β-glucosidase is an enzyme present in honey bee secretions that are responsible for hydrolyzing glycosidic toxins ingested by honey bees converting them to β- glucans, oligosaccharides and glucose, which is a major component of honey.[25] The anticancer effect of cyanogenic glycosides has been suggested to occur by the selective hydrolysis of cyanogenic glycosides by β-glucosidase, liberating cyanide and benzaldehyde at the neoplastic site.[14] The presence of heterodendrin and lotaustralin in *A. dorsata* honey may be one of the factors that contributed to its observed cytotoxic effect.

Sphingolipids are considered as intracellular signal mediators that are involved in many functions such as cell proliferation, differentiation, and apoptosis.[26] Phytosphingosine and sphinganine (Figures 8 and 9) are sphingoid bases that are structural analogs of sphingolipids which are known to play important roles in apoptosis in many cell lines.[27] Nagahara et al.[26] provided evidence that phytosphingosine induces apoptosis in human T-cell lymphoma, as shown by chromatin DNA fragmentation, a hallmark of apoptosis. Sphinganine is a sphingosine analog that mediate apoptosis by inhibiting protein kinase C (PKC) and Akt which are important pro-growth signal kinase that respond to a variety of extracellular growth signals.[28,29]

The sphingoid base sphinganine that is putatively present in *A. dorsata* honey may in part be responsible for the observed cytotoxicity in A549 cells as the Akt signaling pathway influence caspase activation and eventually, apoptotic cell death.[27] Another mechanism by which these two sphingolipids can induce apoptosis is through the lysosomal proteases called cathepsins (Figure 5). According to Kagedal et al.,[30] sphingosine causes relocation of these proteases from the lysosomal compartment to the cytosol which in turn, activates the apoptotic cascade directly by proteolytic activation of the procaspase *Bid.* The presence of phytosphingosine in both the *A. dorsata* honey sample used in this study and that of Dalet et al.[6] suggests that phytosphingosine indeed plays an important role in the cytotoxic effect of *A. dorsata* honey.

The dose-dependent potentiation of the cytotoxic effect of CP to A549 cells by *A. dorsata* honey as observed in Phase I may also be attributed to the specific bioactive compounds present in *A. dorsata* honey. It may be possible that a certain component in *A. dorsata* honey, in low amounts, predominates and causes the observed upregulation in *CYP2J2* which is relatively higher than *CYP1B1* and thus, an increase in cytotoxicity to A549 cells (Figure 1). Hypothetically, for example, sphingolipids which is present in low amounts in the sample may have possibly caused the activation of *CASP8* and thus, led to the observed increase in cytotoxicity in Figure 1. However, if a higher concentration of honey is used, there might be other components from the honey with other effects that becomes predominant compared to the sphingolipids thereby reducing its effect which leads to a rather different dynamics in the expression of *CYP450* genes. It must be noted that natural honey is a complex mixture of various bioactive compounds with varying therapeutical activities that may promote either pro-survival or pro-apoptotic genes. Therefore, the relative amounts of these bioactive constituents may also be a factor that can influence its ability as an adjunct to chemotherapeutic drugs.

The findings of this study may be enhanced by addressing the following limitations during the study. First, the absence of western blot analysis post-qPCR, which could have provided insights into gene expression at the protein level. Additionally, it would be beneficial to conduct animal testing to evaluate the efficacy of honey as a supplementary treatment alongside cyclophosphamide therapy. Lastly, it would also be valuable to describe the kinetics of the putatively identified compounds through LC-MS, following molecular docking analysis.

CONCLUSION

In this study, *Apis dorsata* honey, in low concentrations, was shown to enhance the cytotoxic effect of cyclophosphamide (CP) in A549 cells as previously established. Gene expression analysis of *CYP2J2* and *CYP1B1*, revealed a significant upregulation reaching up to 2.38-fold and 1.49-fold, respectively as compared to untreated cells. This suggests that at a low concentration of *A. dorsata* honey, cytotoxicity in A549 cells is favored due to *CYP2J2* being relatively more upregulated than *CYP1B1*. This cytotoxic effect is further enhanced by a 1.65-fold upregulation of the proapoptotic gene *CASP8* that is paralleled by a downregulation of the antiapoptotic gene *BCL2* as compared to untreated cells. The sphingoid bases phytosphingosine and sphinganine are notable honey constituents that may be linked to the increased cytotoxicity of CP observed in A549 cells due to their role in promoting apoptosis. While *A. dorsata* honey potentiates the cytotoxic effect of cyclophosphamide in a dose-dependent manner, it is further recommended to determine the gene expression profiles of *CYP2J2* and *CYP1B1* in A549 cells treated with higher concentrations of *A. dorsata* honey in combination with cyclophosphamide to confirm its ability to modulate the expression of the aforementioned genes.

Moreover, it is also recommended to perform western blot analysis and in vivo animal testing.

Acknowledgments

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Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

All authors declared no conflicts of interest.

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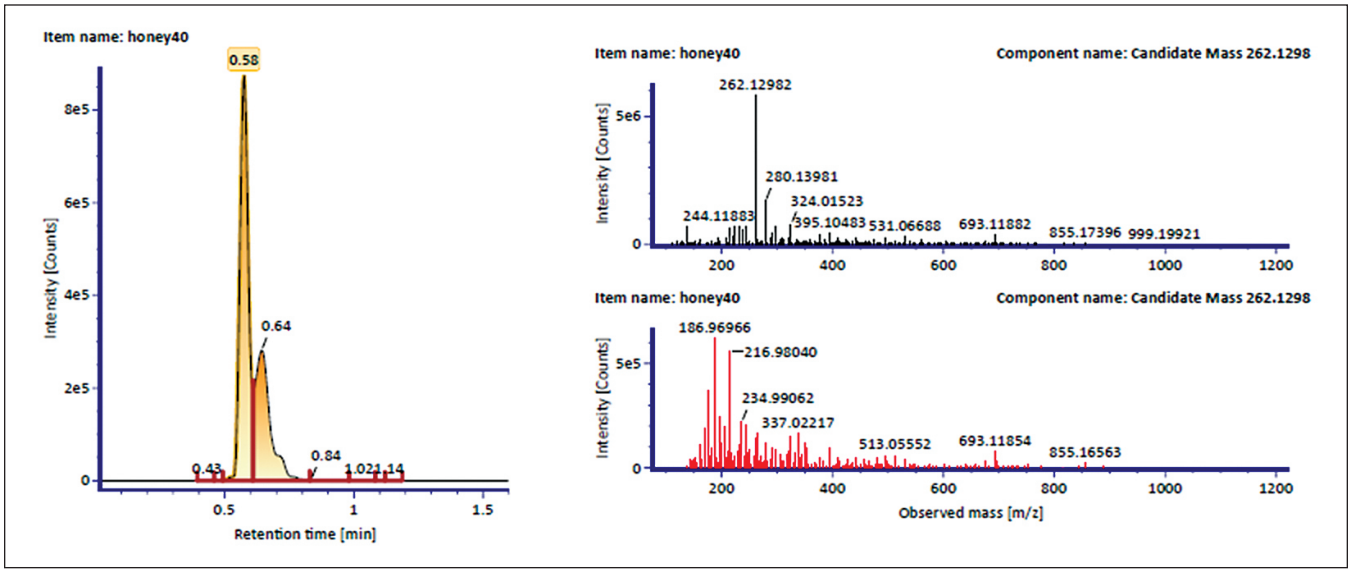
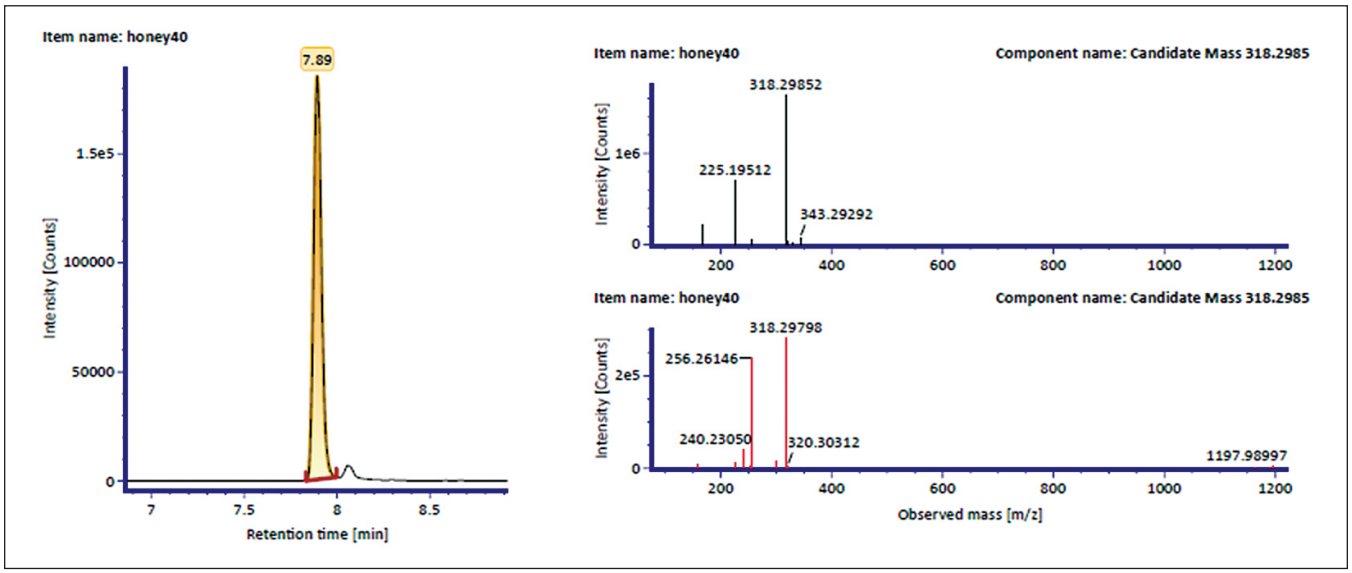
APPENDICES

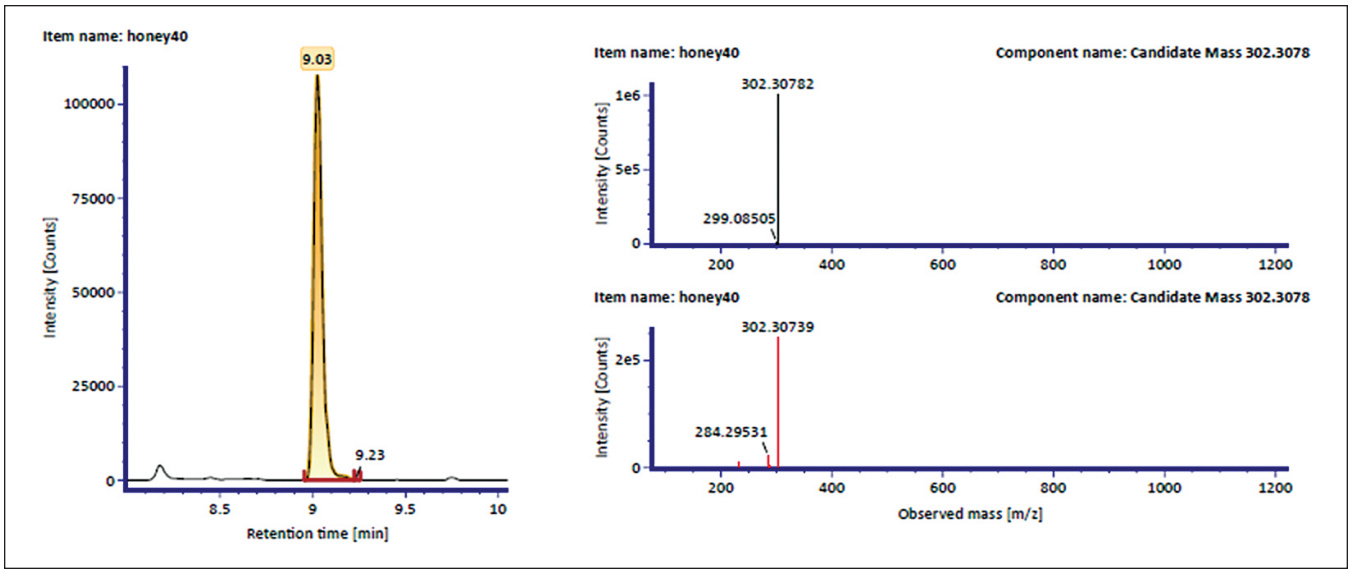
The data from MS were processed through the Waters UNIFI Scientific Information System v1.8.1.073



Appendix Figure 1. Fragmentation pattern of the putative compounds heterodendrin and lotaustralin.



Appendix Figure 2. Fragmentation pattern of the putative compound phytosphingosine.



Appendix Figure 3. Fragmentation pattern of the putative compound sphinganine.