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Agarwood Branch Ethanolic Extract Affects Expression of Apoptotic Genes in MCF-7 Breast Cancer Cells

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Abstract: Breast cancer is one of the most common death causes among women worldwide. Treatment is usually associated with chemically synthesized drugs with serious side effects which shifted the attention of cancer researchers towards development of natural product alternatives. Ethnopharmaceutical evidence showed that *Aquilaria* spp. have been used to treat a wide range of disorders. However, scientific evidence is still lacking to support and extend the traditional applications to cancer. This study aims to investigate differential gene expression (DEG) of MCF-7 cells treated with agarwood branch ethanolic extract (ABEE) to provide insights into its cell growth-inhibiting effects. Methods: cDNA synthesis from RNA of MCF-7 cells treated with the 8 µg/ml ABEE and DMSO-treated cells (control), respectively, were subjected to RT2 Profiler Array Human Cell Death Finder™ containing 84 genes related to cell death mechanism. Pathway analysis was carried out using the online KEGG Pathway tool. Results: 48 genes that met the threshold fold regulation cut-off of 2 and $p < 0.05$; 41 DEGs from the list were down-regulated, and 7 were up-regulated. Pathway analysis suggested ABEE may have caused apoptosis of MCF-7 cells through extrinsic and/or intrinsic apoptotic pathways, including activation of p53 that could be the first step towards apoptotic elimination of the cancer cells upon treatment of ABEE. Conclusion: Results obtained supported the growth inhibition effects of ABEE against MCF-7 cells that can serve as the basis for further work towards extending the use of agarwood as cancer therapeutics.

Keywords: Agarwood; breast cancer; growth inhibition; gene expression; RT2 profiler array

1. Introduction

Cancer is a global challenge with a high mortality rate and an estimated death toll of 9.6 million in 2018[1,2]. The most common cancer listed by the World Health Organization (WHO) is breast cancer with 2.1 million cases and 627,000 death worldwide[3]. Asia recorded 43.6% of new cases of breast cancer with 310,577 death in 2018[4]. The Malaysian Study on Cancer Survival by the National Cancer Registry, Ministry of Health Malaysia (2018) reported the age pattern for female breast cancer showing 34.9% of cases from the 45–54 years and the 5 year relative survival of contracting is 76.5 % for Chinese women, 70.5% for Indian women, and 57.9% for Malay women[5].

Cancer treatment is usually associated with chemically synthesized drugs with increasing evidence of detrimental effects on patients' post-treatment life and well-being. Programmed cell death, consisting of three main forms (apoptosis, autophagy, and necrosis) is the center of attention where it is not only a principle mechanism of tumor suppression but also is activated in non-malignant cells to defeat unnecessary, aged, and damaged cells[6]. In regular conditions, apoptosis acts as a defense mechanism to prevent cancer and tumorigenesis. Any impairment in the apoptotic pathway leads to the formation, progression, and metastasis of cancer cells that could subsequently lead to treatment resistance[7,8]. The activation of the apoptotic core system is evoked by response towards cancer therapy[9]. A nonsurgical means of cancer treatment is conclusive via activation of apoptosis. Hence, a profound understanding of the apoptosis mechanism and its faulty status will assist in producing apoptosis-targeted therapeutics[10].

The undesired health issues associated with the use of chemotherapy drugs have shifted the attention towards using natural products as alternative substitutes. Natural products derived from natural resources such as plants and animals have been traditionally used for centuries by local folks to treat illnesses, including cancer[11]. In this present study, local species of agarwood were investigated as an alternative for cancer treatment. Agarwood which is also known as Gaharu (Malay) or aloeswood is formed in the diseased tree of genus *Aquilaria*, in the family of *Thymelaeaceae*[12]. The fragrant resinous heartwood has been traded since ancient times and is still being used to fulfill demand in religious, medical, and aromatic preparation[13]. According to Barden *et al*.[14], agarwood literature has been found in Sahih Muslim dated back to the eighth century, *Old Testament*, and in the Ayurvedic medicinal text known as the *Susruta Samhita*.

Traditionally, agarwood has been used as a sedative, analgesic, and digestive medicine[15]. Scientific works on the pharmacological activities of Aquilaria spp against cancer and inflammatory have been documented as early as 1981 by Gunasekera *et al*.[16] where an alcoholic extract of *Aquilaria malaccensis* stem bark has been found to have mild cardiotonic activity and anti-cancer against Eagle's carcinoma, which a type of nasopharyngeal carcinoma that usually occurs in the nasopharynx located behind the nose and above the back of human throat. While traditional use was more restricted to the agarwood infiltrated resin, reports in more recent years showed various biological effects of different parts of agarwood trees, including leaves, fruits, seeds, bark, and branches. It is

noteworthy that the formation of agarwood infiltrated resin is a unique phenomenon where it only occurs in diseased trees as part of the systemic defense. The trees could be diseased either by a natural cause such as by lightning or induced by physical means (axed) or using inoculation concoction using certain techniques. Therefore, materials from agarwood trees could also be classified further to those obtained from non-inoculated (non-infected) trees (as in the case of this current study) and inoculated (infected) trees, respectively. Materials obtained from the two types of trees could give different effects on the biological system tested. In Malaysia, the new trend of cultivating agarwood trees in plantation schemes has become more popular to fulfil the increase in agarwood demand and this practice is also growing all around the world, including China and Bangladesh[17–19]. This new scheme provides an unlimited supply of raw materials ready for research and development at a cheaper price.

Recent prominent findings on the pharmacological activity of agarwood from various species and sample types as antioxidant, antimicrobial, antiangiogenic, anticancer and antitumor. A study reported ethanol extracted *Aquilaria crassna* stem bark showing cytotoxicity against several cancerous cell lines, including HCT116, PANC-1, PC3, and MCF-7[20]. Another study also reported potent cytotoxicity against HC116 cells by the *Aquilaria malaccensis* stem bark extract[21]. Additionally, the leafy part of the agarwood tree was reported to exhibit potential antioxidant[22,23] and laxative activities[24,25]. Among potentially active compounds reported in the agarwood trees in relation to biomedicinal effect includes phenolic acid (4-hydroxybenzoic acid, palmitic acid, and 9-octadecanoic acid), terpenoids (cucurnitacins, aquilanol A, aquilanol B, and β-agarofuran), phenols (hydroquinone, 4-hyhdroxyacetanilide, and phenol, 2,6-dimethoxy), flavonoids (aquilarinoside A1, 5-hydroxy-4', 7-dimethoxyflavone, and 3'-hydroxy genkwanin, aquilarinoside A1), steroid (stigmasterol, β-sitosterol, and β-sitostenone), fatty acid esters, fatty alcohols, alkanes and others[26–32]. These bioactive compounds may be responsible for the biomedicinal effects reported many studies.

Previous data from our laboratory showed that agarwood ethanolic branch extract from *Aquilaria subintegra* (non-inoculated tree) was able to inhibit the growth of MCF-7 breast cancer cells with the inhibition concentration (IC50) value of 8 µg/ml (final concentration in growth medium) after 72 hours of treatment[21]. In the study, MCF-7 cells were observed to be shrinking and showed signs of pyknosis as compared to control cells during treatment. Subsequent cytokinetic study of the same sample at the same concentration showed an increase in the death rate of cancer cells[33]. Therefore, to further study the mechanism of anti-cancer and cytotoxicity activities of *Aquilaria subintegra* an investigation by using a differential gene expression approach was conducted.

In this present study, the RT2 Profiler PCR Array was used to determine the differential genes that are known and thought to be associated with the progression of cancer cells. The RT2 Profiler PCR Array takes advantage of the combination of real-time PCR performance and the ability of microarrays to detect the expression of many genes simultaneously. High-throughput gene expression data obtained from microarrays are

interpreted in pathway analysis that integrates diverse biological information concerning specific diseases[34]. This can provide insights into the mechanism of gene regulation and at the same time develop a comprehensive pathway knowledge-based system based on agarwood-treated breast cancer. The understanding of the underlying pathways of the cytotoxic effects of the extract may help to explain the likely effects and role of agarwood in breast cancer therapeutics. To the best of our knowledge, this is the first study on differential expression of an array of genes associated with breast cancer following treatment of agarwood material.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Dulbecco's modification of Eagle's medium, DMEM (with high glucose and L-glutamine) in powder form, and fetal bovine serum (FBS) were supplied by Gibco™, USA. Cell detachment enzyme known as Accutase was obtained from Innovative Cell Technologies, USA. RNeasy Mini kit, RT^2 First Strand kit, and the RT^2 Profiler Array kit were obtained from QIAGEN, Germany. Dimethylsulfoxide (DMSO) was obtained from Amresco, USA.

2.1.2 Cell lines

MCF-7 breast adenocarcinoma cell (ATCC® HTB-22™) was obtained from American Type Culture Collection (ATCC).

2.1.3 Plant

Branches of un-inoculated trees of *Aquilaria subintegra* with an average age of 5 to 6 years were obtained from a local Malaysian plantation located in Bangi, Selangor, Malaysia. The branches were identified according to their morphology and voucher specimen #HBL707[VS-2] was deposited at KAED Herbarium at the International Islamic University Malaysia. The un-inoculated trees refer to healthy trees that have not gone through manual inoculation of fungus or bacterial concoction to imitate the agarwood formation process. The raw materials were freshly collected, washed, rinsed, and dried before being pulverized into a fine powder for the solid-liquid extraction process. The powder was stored in air-tight containers until further use.

2.1.4 Agarwood ethanolic extract

The sample was extracted using absolute ethanol (HmbG Chemical, Germany) with the following parameters: extraction time of 24 hours, the temperature of 50°C, agitation speed of 200 rpm, and 1:20 (w/v) solid to solvent ratio as reported by Abbas *et al*.[17]. After incubation, the extracts were filtered and evaporated using a rotary evaporator to obtain the

alcohol-free agarwood extract. Dried agarwood branch ethanolic extract (ABEE) was prepared as a stock solution using 100 % (v/v) dimethylsulfoxide (DMSO) and then diluted using deionized distilled water to adjust to the final concentration of 8 µg/ml. This final concentration of 8 µg/ml was the observed IC_{50} ABEE against MCF-7 cancer cells using sulforhodamine B (SRB) assay as reported in our previous study (Abbas *et al*., 2017)[17,33]. Our previous study also included Taxol as the positive control set as comparison to agarwood tested group which showed much severe inhibition of MCF-7 cancer cells with IC_{50} of 2.8 µg/ml[33]. Also included previously was the effect of ABEE against VERO cells (normal cell representation) that showed milder inhibition (14-49 µg/ml)[17].

2.2 Methods

2.2.1 ABBE treatment of MCF-7 cells

Confluent MCF-7 cells were sub-cultured into a set of T-75 cm^2 flask at a seeding concentration of 1.5 x 10^6 cells in 15 ml of medium (DMEM and FBS ratio 9:1) and incubated at 37°C in a 5% CO_2 humidified atmosphere and 95 % air for 24 hours. Dilution of 8 µg/ml Agarwood branch ethanolic extract (ABEE) was then introduced to the cells during the medium change, and the flasks were incubated for further a 24, 48, and 72 hours, respectively. Morphology changes, cell viability, and cell count after treatment were observed using an inverted phase-contrast light microscope (Olympus, USA). Cells were harvested and counted using the trypan blue dye exclusion method at a pre-determined time interval (24, 48, and 72 hours). Cells were pelleted by centrifugation and were stored at -80°C until further use.

2.2.2 RNA Extraction and cDNA synthesis

For gene expression study, cells treated for 48 hours were used considering that it gives an adequate number of cells for quality gene expression work while also ensuring that the effect of treatment has taken place. Total RNA was extracted from the cells by using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's recommendations. RNA concentration and integrity values were analyzed using a bio-photometer (Eppendorf, Germany). For this study, the starting amount of RNA was set at 0.5 µg, and the cDNA synthesis was conducted using the QIAGEN RT^2 First Strand cDNA synthesis kit (Qiagen, USA).

2.2.3 RT^2 Profiler PCR Array

Human Cell Death PathwayFinder™ RT Profiler™ PCR Array (PAHS-212Z) in combination with RT^2 SYBR® Green qPCR Mastermix (Qiagen, USA) was used to screen a panel of 84 pathway-specific genes related to cell death. The PCR Array was conducted under the following conditions: 95°C for 10 min, then 40 cycles at 95°C for 15 sec, and 60 °C for 1 min. Each array contained five separate housekeeping genes (ACTB, B2M, HPRT1, GAPDH, and RP1P0) that were used for the normalization of the sample data. Cycle

threshold (C_t) values, fold changes of gene expression, scatterplot and heatmap were analyzed and generated by using RT^2 PCR array data analysis web portal version 3 available online at http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php.

2.2.4 KEGG Pathway analysis

The Pathview library of the bioconductor was used to generate the apoptosis signaling pathway and necroptosis signaling pathway[34]. The fold values of significantly changed genes were mapped by colors on native KEGG, apoptosis signaling pathway (KEGG ID = hsa04210), and P53 signaling pathway (KEGG ID = hsa04115), where green represents down-regulated expression and red represents up-regulated expression levels normalized to the control group[35,36]. To show a comprehensive image concerning the regulation of the analyzed signaling pathways, all genes whose expression was significantly different without a cut-off at fold values were visualized.

3. Results and Discussion

3.1. Effect of ABEE Treatment on Cell Viability and Morphology

The branch extracts (ABEE) at 8 µg/ml final concentration inhibited the growth of breast cancer, MCF-7 cell lines eventually leading to cell death. Starting with 1.5 x 10^6 cells, cell numbers were measured after 24 h, 48 h, and 72 h. The number of cells was substantially reduced in the treated compared to untreated flasks. Figure 1 shows the comparison of cell viability between control and treated cells at three-time intervals (n = 3 ± s.d.); with the highest cell viability reduction of 74.36 ± 6.51 % of control at 72 h (1.0 x 10^6 viable cells in the treated group compared to 3.9 x 10^6 cells in the control group). Viable cell reduction of 26.67 ± 12.80 % at 48 hours and 28.48 ± 9.26 % at 24 hours were also observed and recorded. This effect of ABEE observed on the viable cell numbers might be due to the induction of apoptosis in cells, but it might as well be due to the effect of ABEE initiating cell proliferation arrest as an emergency program.

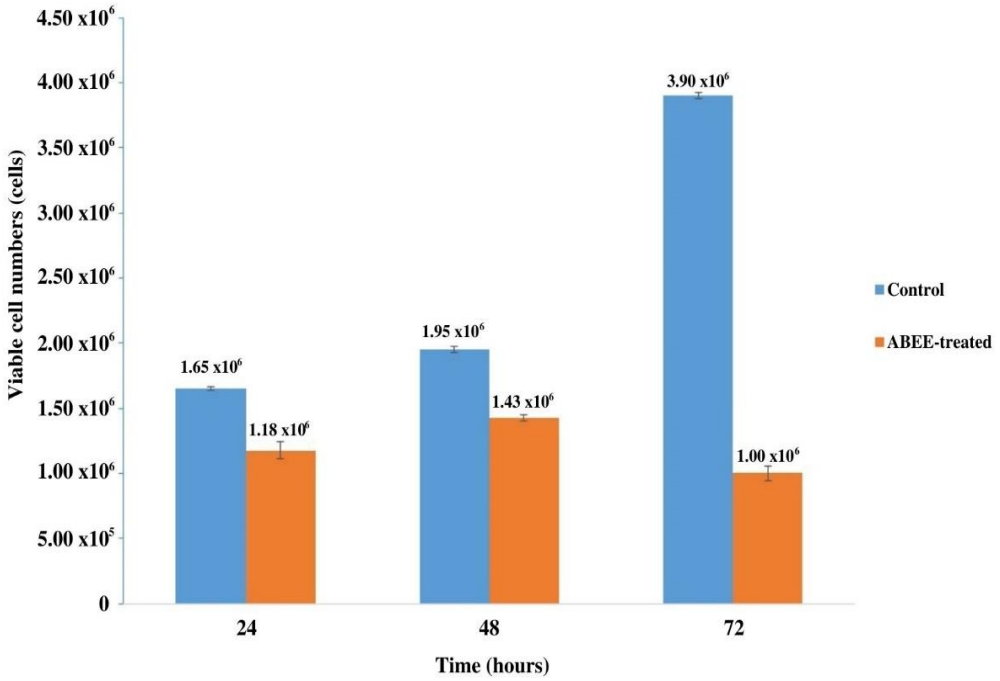


Figure 1. Cell viability at 24, 48, and 72 hours of treatment of 8 µg/ml Agarwood branch ethanolic extract (ABEE) as compared to control (0.1 % DMSO v/v). Results are based on three independent experiments (*n* = 3 ± s.d.). Cell viability percentage reduction observed were 28.48 %, 26.67 %, and 74.36 % for the 24, 48, and 72 hours, respectively.

Figure 2 shows the morphology of MCF-7 cells observed under an inverted phase light microscope at 24, 48, and 72 hours for both control and treated groups, respectively. Treated cells started to show some abnormalities in morphology where they were observed to be shrinking only after 48 hours. This is in agreement with earlier studies by Abbas *et al*.[17,33,37], thus reaffirm the cytotoxicity effects of ABEE at the same IC_{50} and enable the proper selection of the most suitable time for cell harvesting to fulfill the minimum RNA requirement for the gene expression study. The essential requirement for apoptosis is to be rapid and must be highly efficient to evade residual malignant cells. For instance, FasL or TRAIL ligand initiates apoptosis within a few hours where the majority of cells were found dead on microscopic examination after 24 hours[35]. In this study, cells appear to have abnormal morphology following ABEE treatment, with some degree of reduction of cell number (Figure 1 and 2). This observation depicts the feature of cell death with a spectrum of apoptotic morphology but with a delayed time of death. The delayed time of death refers to the ABEE-treated cells which shows viable cells with some degree of morphologic abnormality prone to apoptosis (but still viable at 24 hours). The morphologic abnormality was observed to be affecting more viable cells at 48 and 72 hours accompanied by reduced cell population density. Gene expression analysis was therefore employed using the "cell death pathway finder" to analyze the gene expression profile of MCF 7 cells at 48 h after being treated with ABEE, and elucidate the potential pathways or mechanisms involved.

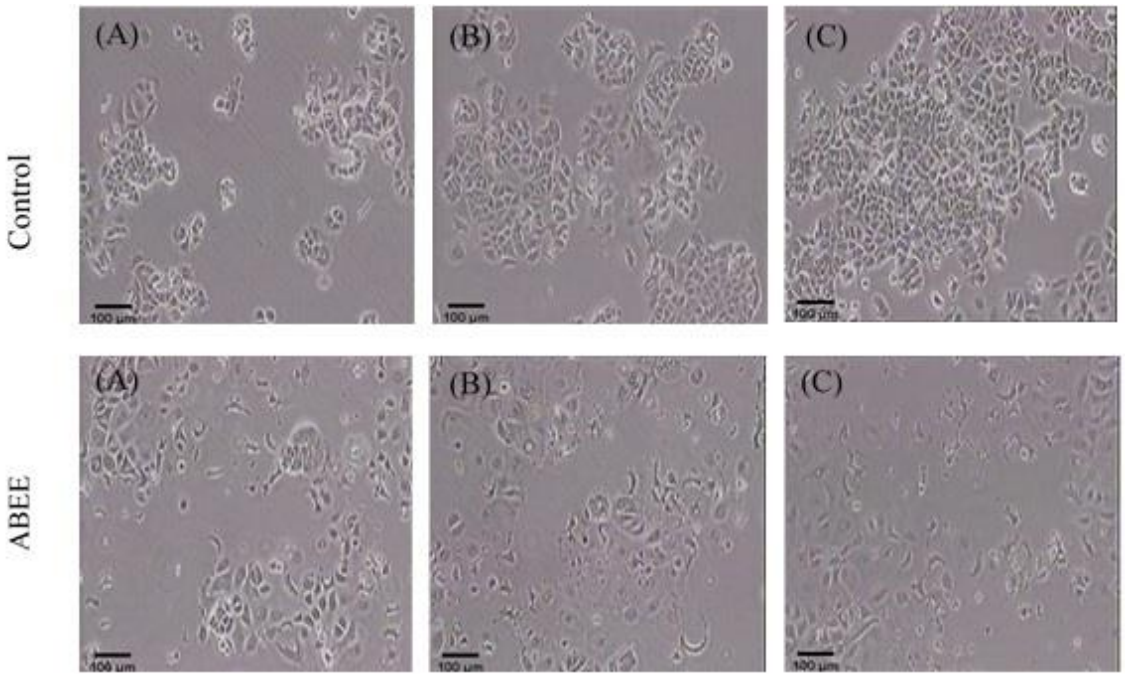


Figure 2. Representative images of MCF-7 cells population density at (A) 24, (B) 48 and (C) 72 hours. The treated group showed abnormal morphology in all-time points while the control group showed normal morphology. The lowest growth population density was observed at 72 hours ABEE-treated group with clear irregular cell morphology compared to control group at the same time point.

3.2 Gene Expression Profiling

The gene expression of agarwood branch ethanolic extract treatment on MCF-7 for 48 hours was analyzed using a real-time PCR array containing 84 genes associated with apoptosis, necroptosis, and autophagy. Based on the previous cytokinetic study of ABEE against MCF-7 cells, the changes in the population density of cells were observed as early as 24 hours after incubation and became more apparent at 72 hours with the presence of shrinking and floating cells (Abbas *et al*., 2017). The differential gene expression level of the 84 genes involved in the common cell death pathway was assessed. A number of 48 genes with fold change cut off 2 ($p < 0.05$) across all samples are presented in Table 1. Based on significant genes, the expression of downregulated genes was between 2.18 to 26.704, while the range for upregulated genes was much smaller (2.23 to 5.52). NFKB1 was the most downregulated with the largest magnitude of fold regulation (26.704) while TRAF2 was the most upregulated with fold regulation of 5.52.

Table 1. List of genes with fold change cut off 2 ($p < 0.05$)

Gene symbol	Fold regulation	Gene symbol	Fold regulation
NFKB1	-24.704	**CCDC103**	-3.648
HSPBAP1	-22.911	**ESR1**	-3.468
HTT	-19.266	**BIRC2**	-3.046
TNF	-18.94	**DEFB1**	-2.99
CASP7	-10.296	**EIF5B**	-2.977
APAF1	-9.87	**APP**	-2.973
IGF1	-8.83	**BCL2L11**	-2.898
CASP9	-7.868	**FOXI1**	-2.711
AKT1	-7.695	**ATG16L1**	-2.698
ABL1	-7.637	**CASP2**	-2.694
DFFA	-6.169	**CASP3**	-2.691
GALNT5	-5.873	**PARP2**	-2.681
INS	-5.594	**CASP6**	-2.585
BCL2L1	-5.408	**MAPK8**	-2.393
CTSS	-5.102	**ATG7**	-2.355
S100A7A	-4.81	**PARP1**	-2.318
COMMD4	-4.787	**NOL3**	-2.18
BCL2	-4.747	**CTSB**	2.23
ATG12	-4.727	**TP53**	2.41
ATG3	-4.573	**RPS6KB1**	2.55
ATP6V1G2	-4.287	**MAG**	2.57
SYCP2	-4.252	**TXNL4B**	2.64
DPYSL4	-3.964	**BMF**	3.9
BAX	-3.722	**TRAF2**	5.52

Figure 3 shows the scatter plot that compares the normalized expression of every gene on the array that was plotted against one another to visualize large gene expression changes. The central line indicates the unchanged gene expression. The dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold. It is clear that more genes are being downregulated upon treatment of ABEE as compared to upregulated genes.

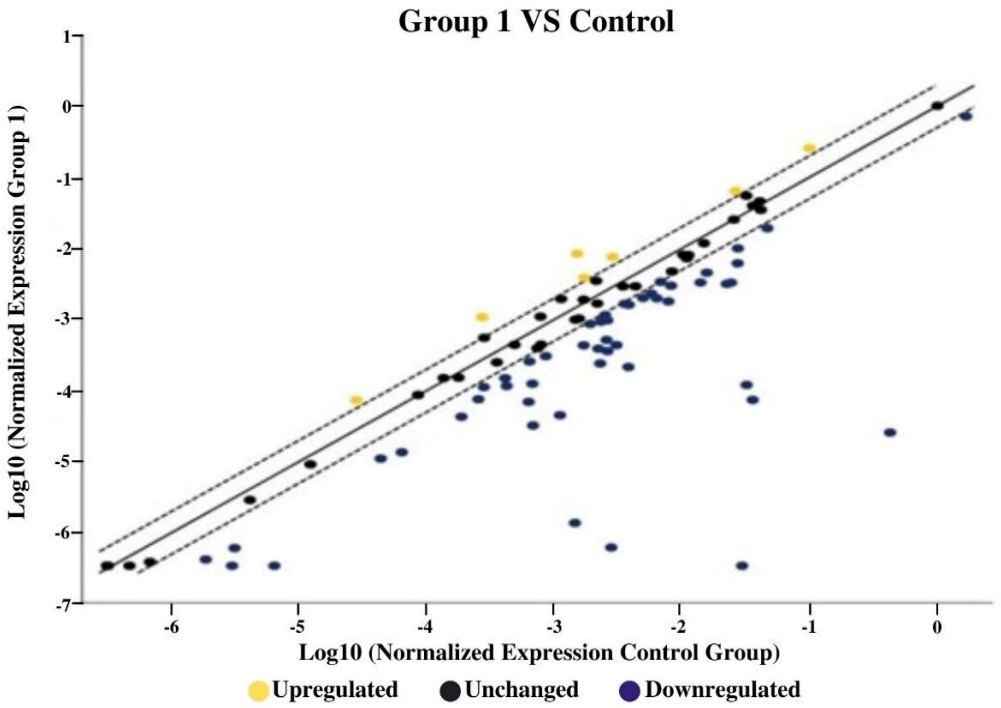
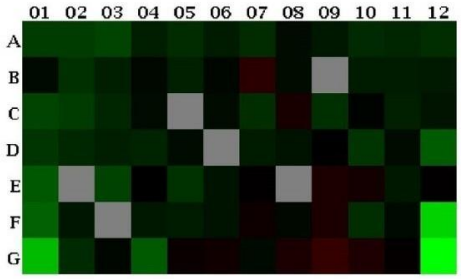


Figure 3. Scatter plot of normalized expression of cells treated with ABEE between normalized control groups. This scatter plot compares the normalized expression of every gene on the array that was plotted against one another to visualize large gene expression changes. The central line indicates unchanged gene expression while dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold.

A heat map provides a graphical representation of the expression of the ABEE treated genes against the control group in the whole array. In Figure 4, red represents an upregulated gene expression while the green color represents a downregulated gene expression.

(A)



(B)

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ABL1 -7.12	AKT1 -7.18	APAF1 -9.20	APP -2.77	ATG12 -4.41	ATG16L1 -2.52	ATG3 -4.26	ATG5 -1.50	ATG7 -2.20	**ATPV1G2 -4.00**	BAX -3.47	BCL2 -4.43
B	**BCL2A1 -1.36**	BCL2L1 -5.04	BCL2L11 -2.70	BECN1 -1.31	BIRC2 -2.84	BIRC3 -1.18	BMF 3.90	C1orf159 -1.48	**CASP1 1.07**	CASP2 -2.51	CASP3 -2.51	CASP6 -2.41
C	CASP7 -9.60	CASP9 -7.34	CCDC103 -3.40	CD40 -1.44	**CD40LG 1.07**	CFLAR -1.53	COMMD4 -4.46	CTSB 2.23	CTSS -4.76	CYLD -1.12	DEFB1 -2.79	DENND4A -1.89
D	DFFA -5.75	DPYSL4 -3.70	EIF5B -2.78	ESR1 -3.23	FAS -1.44	**FASLG 1.07**	FOXI1 -2.53	GAA -1.78	GADD45A 1.00	GALNT5 -5.48	GRB2 -1.52	HSPBAP1 -21.36
E	HTT -17.96	**IFNG 1.07**	IGF1 -8.83	IGF1R -1.01	INS -5.22	IRGM -1.77	JPH3 1.09	**KCNIP1 1.07**	MAG 2.57	**MAP1LC3A 1.94**	MAPK8 -2.23	MCL1 1.10
F	NFKB1 -24.90	NOL3 -2.03	**OR10J3 1.07**	PARP1 -2.16	PARP2 -2.50	PIK3C3 -1.80	PVR 1.62	RAB25 -1.27	RPS6KB1 2.55	S100A7A -4.48	SNCA -1.39	**SPATA2 -1068.23**
G	SQSTM1 -489.44	SYCP2 -3.96	TMEM57 -1.19	TNF -18.94	**TNFRSF10A 1.39**	**TNFRSF11B 1.70**	TNFRSF1A -1.41	TP53 2.41	TRAF2 5.52	TXNL4B 2.64	ULK1 1.11	**XIAP -4547.95**

Figure 4. (A) Heat Map and the (B) table provides the fold regulation data used to visualize the fold changes in expression between the selected groups for every gene in the array in the context of the array layout. Expression levels above the mean for the gene are shown in red squares and expression levels below the mean for the gene are shown in green squares.

Altogether 48 genes meet the threshold cut-off 2 where 41 differentially expressed genes from the list were significantly down-regulated, and the remaining 7 were significantly up-regulated (Figure 5). Changes in the expression of these genes are generally indicative of cellular responses to stimuli of specific apoptotic signals.

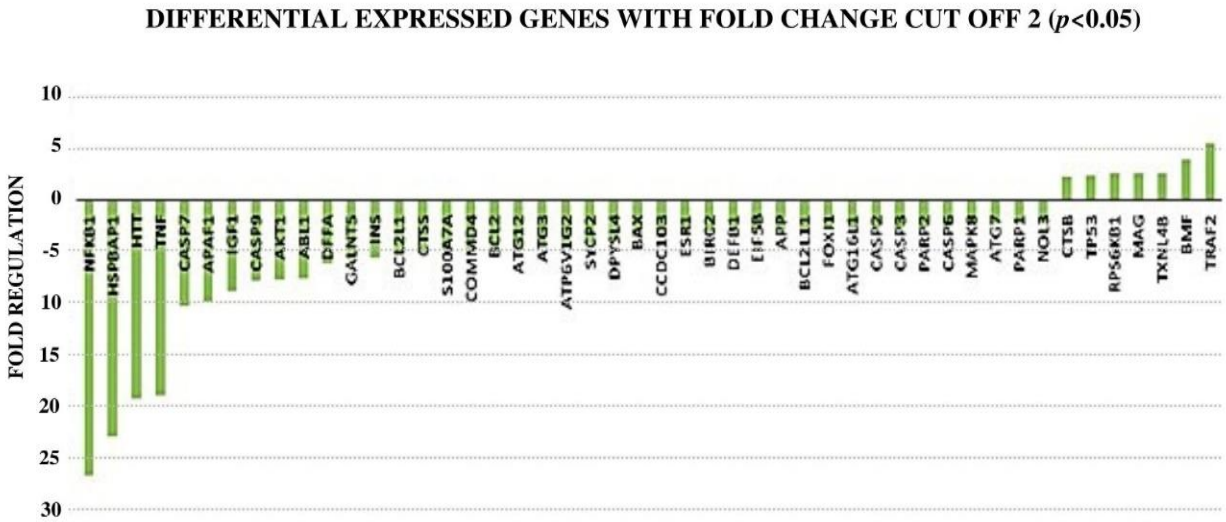


Figure 5. Differential expression gene after the ABEE treatment on breast cancer cell with fold change cut off 2 and significant difference at $p < 0.05$ versus control.

3.3 Pathway Analysis

In general, the effects of ABEE treatment on MCF-7 cell number and morphology indicated the involvement of apoptotic pathways. Apoptosis is a programmed cell death (PCD) that is regulated in a multicellular organism associated with atypical morphologic changes, including cell shrinkage, chromatin condensation, and cytoplasmic blebbing[39]. Apoptosis is regulated through two significant pathways in mammalian cells. First is the extrinsic pathway that is initiated through the triggering of the tumor necrosis factor family (TNF) followed by the binding of the FAS-associated death domain to the death receptor containing the intracellular death domain (FADD) protein[40]. The second is the intrinsic pathway that can be activated by physical and chemical stimulation. Both pathways resulted in proteolytic cleavage and activation of effector caspase (Caspase 3). Results from this study show that genes that are required for the activation of programmed cell death were either markedly downregulated or unaltered, such as TNF, caspases, Fas, and TNFR.

Figure 6 shows the apoptosis pathways affected when MCF-7 cells were treated with ABEE. It can be seen that both extrinsic (receptor-mediated) pathways and intrinsic (mitochondria-mediated) pathways were involved.

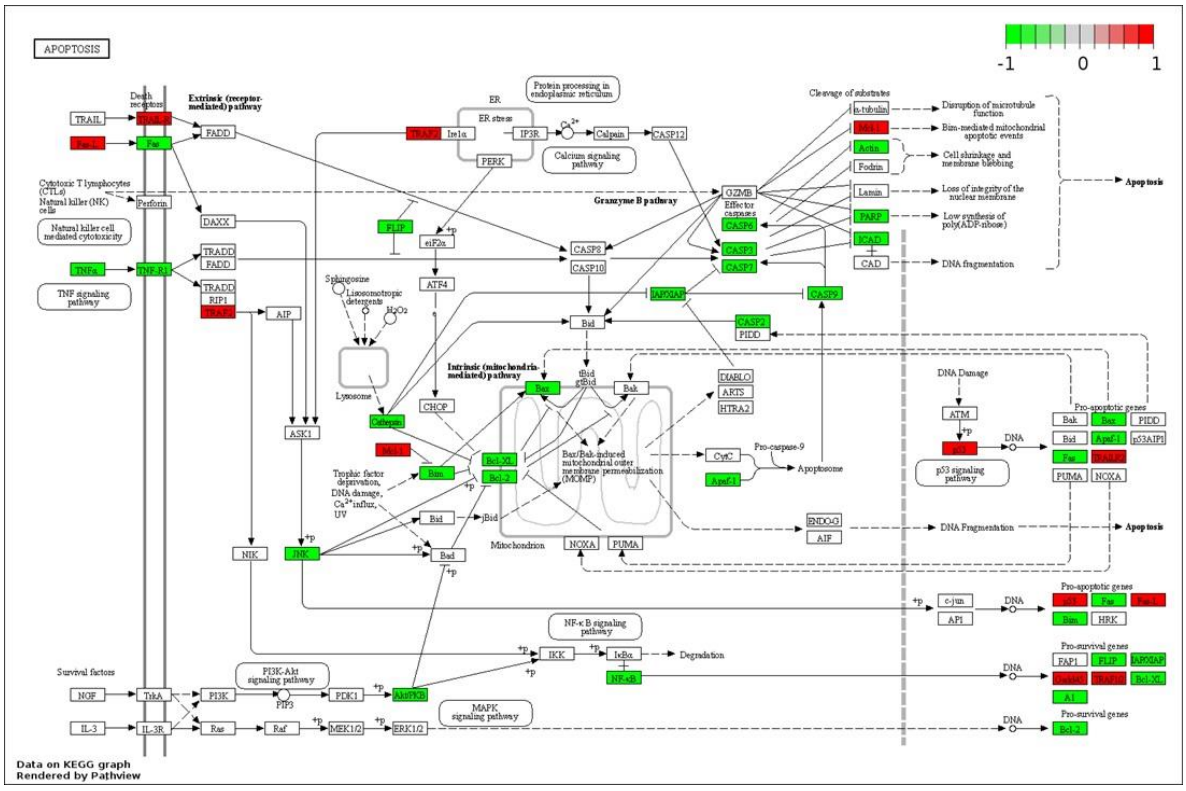


Figure 6. Apoptosis pathway in MCF7 cells treated with ABEE. Expression changes of target genes are mapped by colors; red color—statistically significant increase in expression, green color—statistically significant decrease in expression. Green colored nodes: Tumor necrosis factor α (TNFα), tumor necrosis factor receptor 1 (TNF-R1), Fas receptor (Fas), FLICE inhibitory protein (FLIP), Jun N-terminal Kinase (JNK), Cathepsin B (Cathepsin), Bcl-2-like protein 11 (Bim), Protein Kinase B (Akt/PKB), B-cell lymphoma-extra large (Bcl-XL), B-cell lymphoma 2 (Bcl 2), Bcl-2 Associated X protein (Bax), Nuclear Factor kappa-light-chain–enhancer of activated B (NF-κβ), Apoptotic protease activating factor 1 (Apaf-1), X-linked Inhibitor of Apoptosis protein (IAP/XIAP), Bcl-2 survival promoter (A1 or Bfl-1 or GRS). Red colored nodes: Fas ligand (Fas-L), TNF Receptor Associated Factor 2 (TRAF2), MCL1 Apoptosis Regulator (Mcl-1), Tumor Protein 53 (p53), Death Receptor 5 or TRAIL Receptor 2 (TRAILR2), Growth Arrest and DNA Damage-inducible 45 (Gadd45), and TNF Receptor Associated Factor 1 and 2 (TRAF 1/2).

Following the observation of pathways involved in Figure 6, the p53 signaling pathway was further explored. Figure 7 shows the p53 signaling pathway that was generated using the same gene expression result. Activation of the p53 gene triggers several parallel pathways that block the formation of the mitotic cyclin B/Cdc2 complex and inhibit the activity. In this case, Gadd45 will bind Cdc2 and disrupts its ability to complex with cyclin B, therefore, inhibits the cyclin B/Cdc2 activity, which is essential for the cell to enter mitosis.

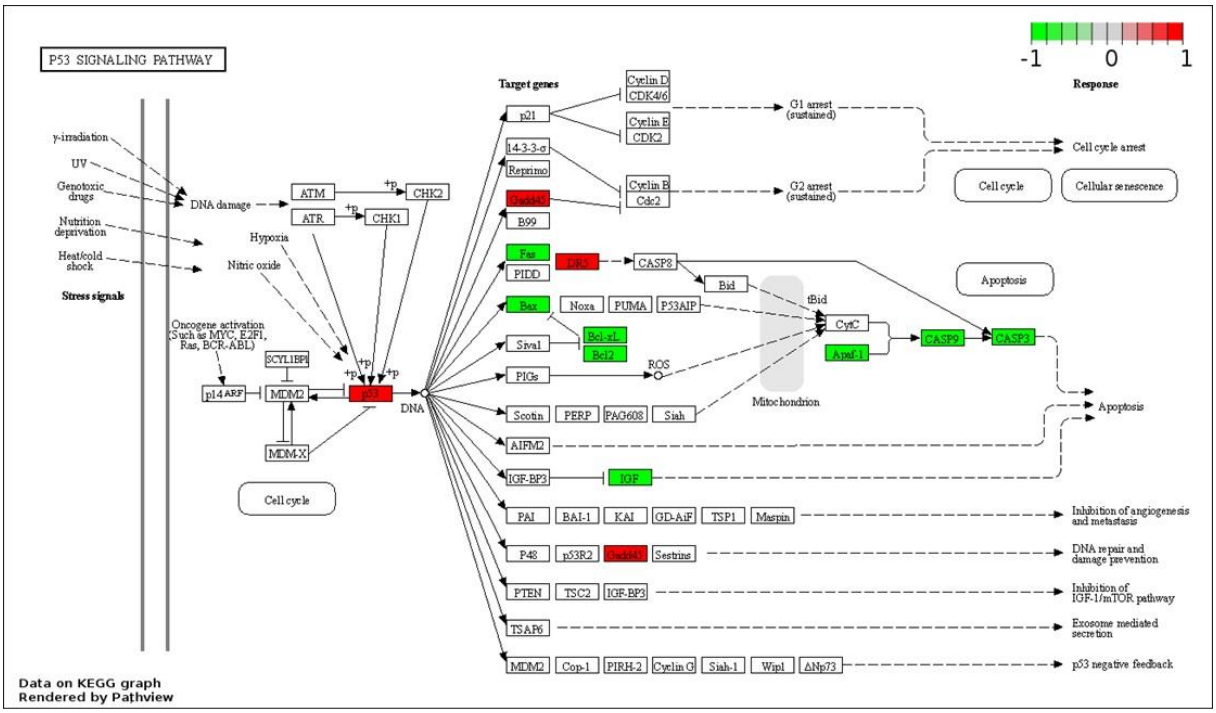


Figure 7. Contribution of p53 to G2 cell cycle arrest. p53 triggers several parallel pathways that block the formation of the mitotic cyclin B/Cdc2 complex and inhibit its activity. Green colored nodes: Insulin-like Growth Factor (IGF), Caspase 9(CASP9), and Caspase 3 (CASP3). Red coloured node: Death Receptor 5 (DR5).

P53 triggers proliferation stops by arresting the cell cycle under different conditions to help cell viability monitoring. If the monitoring found any potential harm toward cellular function, the cell will self-destruct (apoptosis) to prevent further damage to the surrounding environment. It is noted that Bax, Casp and Apaf-1 genes (pro-apoptotic genes) were down regulated upon treatment of ABEE which could be seen as contradicting. However, knockdown of Apaf-1 and caspase-3 has been reported to reduce the cell proliferation not by apoptosis but by G1 and G2/M arrest[41]. In the study, the knockdown of Apaf-1 and caspase-3 in human glioma cells correlated with reduced cell proliferation in which the potential mechanism involved was G1 arrest in A172 cells (human glioma cells) and a G2/M arrest in U118 cells (human brain glioma cells) substantiated by the expression of Cdk2 and p21WAF1/Cip1. After the knockdown, A172 cells appeared to cease proliferation and partially lost their adherence to plastic, and acquired a more spindle-shaped morphology with elongated spikes.

Additionally, the downregulation of Bax, Casp and Apaf-1 was also accompanied by other gene regulations, including FASL, TRAILR2, GADD45, JNK, TRAF2, and several others. As such, it is highly probable that the major apoptotic mechanism in this study starts at the upstream activation of the p53 gene, that triggers several parallel pathways that block

the formation of mitotic cyclin B/Cdc2 complex and inhibit the activity. In this case, the mechanism may be via Gadd45 binding to Cdc2 that disrupts its ability to complex with cyclin B, therefore, inhibits the cyclin B/Cdc2 activity, which is essential for the cell to enter mitosis. Furthermore, the two IAP family of antiapoptotic genes (CIAP-2 and XIAP) which are known to be associated with both intrinsic and extrinsic pathways, were downregulated (see Figure 6). The inhibition of XIAP was shown to increase tumor sensitivity to 5-FU in pancreatic carcinoma cells[42]. To this end, the inhibitory effects and apoptotic characteristics may be due to the sum of all the up and downregulation of related genes upon ABEE treatment. The activation of p53 by ABEE could be the first step towards apoptotic elimination of the cancer cells. The observation that some cells undergo cell death might therefore be a consequence of the p53-controlled quality check[35].

3.4 Roles of Selected Genes in The Apoptotic Pathway

Roles and relationships of the four most down-regulated genes (NF-κB1, HTT, and TNF) and the most upregulated gene (TRAF1) were investigated and discussed in the section below.

3.4.1 NF-κB1

The nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB1) gene was found to be the most statistically significant with fold regulation of -26.704 in cells treated with ABEE. In the extrinsic pathway, NF-κB1 is a heterodimer that functions as a transcription factor that induces inflammatory cytokines and anti-apoptotic proteins[43]. It is associated with resistance to apoptosis, expression of angiogenic proteins, and carcinogenesis due to its fundamental effects on cellular dedifferentiation and proliferation in malignancies[44]. NF-κB1 normally antagonizes program cell death (PCD) by activating the expression of anti-apoptotic proteins and antioxidant molecules. The result shows that the ABEE caused the downregulation of the NF-κB1 gene suggesting inhibition of NF-κB1. Inactivation of NF-κB1 may lead to a significant decrease in cell viability due to apoptosis. This result is in line with studies done by other researchers. For example, inhibition of the NF-κB1 pathway led to potent induction of apoptosis in renal cell cancer[45], bladder cancer[46], and prostate cancer cells[47]. Further, the inactivation of NF-κB1 leads to the inactivation of the AKT 1 gene, an anti-apoptotic gene that promotes cell survival by mediating the cellular growth factor and blocking apoptosis by inactivating pro-apoptotic proteins[48].

3.4.2 HTT

The huntingtin (HTT) gene carries a polymorphic expansion of glutamine (polyQ) in its N-terminal encoded by CAG trinucleotides[49]. HTT, a scaffold protein of 350kDa is well conserved from insects to mammals[50]. HTT gene is needed for normal development and is expressed in most tissues but the normal function of HTT protein is still not clear[51]. The N-terminal of HTT has been extensively studied as the polyQ region is significant in determining the destiny of the gene. The expansion of CAGs trinucleotides decides the complementary pathways, including the regulation of apoptosis, altered transcription, metabolism, and cellular responses[52]. Wild type HTT carriers the expansion of polyQ from 9 to 35 repeats in exon 1 whereas if the repeats exceed 35, the altered protein (mutant HTT) expressed lead to the development of Huntington disease[49]. Huntington's disease is a neurodegenerative disorder with a rate of occurrence of 5–10 individuals per 100,000 in the Caucasian population[53]. The symptoms are usually characterized by a deficit of a combination of three factors which are motor, cognitive and psychiatric symptoms[53].

Apart from being an essential protein in the brain, Huntington proteins are also present in several tissues and are involved in fundamental biological processes[54]. HTT is found to be expressed in both normal and tumor mammary cells. Moreira Sousa *et al*.[55] demonstrated that HTT accelerates tumorigenesis in two mouse breast cancer models. The endosome directly binds dynamin and endogenously expresses HTT. In Huntington's disease, the binding is enhanced, thereby replacing dynamin[56]. The dynamin-dependent endocytosis of human epidermal growth factor receptor 2 (HER 2) tyrosine kinase is reduced, leading to its accumulation and, a subsequent increase in cell motility and proliferation[55]. HER 2 is a gene that can play a role in the development of breast cancer[57]. HTT induces abnormalities in HER 2 endocytosis in the breast cancer cell with consequences on their motility and metastatic behavior[55]. This suggests that downregulation of HTT upon treatment of ABEE could lead to inhibition of breast cancer cell proliferation and metastasis. Plant phytochemicals molecular mechanism effect on HTT role in cancer has not been studied so far as research on HTT has mostly focused on neurological symptoms.

3.4.3 TNF

Tumor necrosis factor (TNF) is involved in regulating the inflammatory microenvironment, tumor progression, the incursion of adjoining tissues, angiogenesis, and metastasis. It is a pro-inflammatory cytokine that plays important role in cell proliferation, differentiation, survival, and death[58]. It is secreted by inflammatory cells and involved in inflammatory-associated carcinogenesis. Interestingly, there is evidence that this protein

displays two distinct functions[59]. First, it displays an important biological function with activation of signaling pathways such as Nuclear factor-κB (NF-κB), a major cell survival signal that is anti-apoptotic and c-Jun N-terminal kinase (JNK), of which its sustained activation contributes to cell death[60]. Aside from that, it can also prevent activation of caspase-8 and thus prevent TNFR1- induced apoptosis. Therefore, TNF can either be an internal tumor promoter and killer at the same time. In this study, the TNF gene was found to be down-regulated, suggesting the activation of the JNK pathway leading to MCF-7 cell death upon treatment with ABEE. Many studies have shown that natural compounds suppressed TNF that further inhibits the expression of NF-κB and IκB kinase in cancer cells[61-63].

3.4.4 TRAF 1

TNF-receptor associated factors (TRAF1) gene is one of the significantly upregulated with the highest fold regulation of 5.52. TRAF1 is involved in signal transduction that mediates cell life and death in the immune response, inflammatory and malignant diseases, and is induced by various cytokines[64]. Many studies report that TRAF1 expression facilitates the survival, proliferation, differentiation, and activation of cancer cells[65]. Expression and even overexpression of this protein occur in many cancer cells[66]. The expression of TRAF1 was found to be upregulated when treated with chemotherapeutic drugs vincristine (cytotoxic compound of the alkaloid class used to treat acute leukemia and other cancers)[64]. Vincristine regulates cytoplasmic localization of polypyrimidine tract binding protein that leads to the internal ribosome entry segment (IRES)-dependent translation of TRAF1. IRES is mainly found within mRNA proteins involved in regulating gene expression during differentiation, cell growth, and apoptosis and become activated under cellular stress and DNA damage. Consequently, activated IRES initiate translation of TRAF1 that mediates signals during cellular stress and apoptosis. This phenomenon was found to be specific for vincristine, and not other chemotherapeutic drugs (DEXA, Dox, MTX). This indicates that not all apoptosis inducer can regulate IRES-dependent TRAF1 translation and it is rather a unique cellular mechanism existed in acute lymphoblastic leukemia cell and other cancer cells that can regulate TRAF1 activity. It is interesting to further look into whether ABEE falls into this group of therapeutics.

4. Conclusions

In this experiment, the expression of 84 genes related to the central mechanism of cell death was profiled and analyzed using RT^2 Profiler Array Human Cell Death Finder™. The aim was to investigate the mechanism of growth inhibition of MCF-7 cells upon treatment of

ABEE. There were 48 genes threshold cut off 2, forty-one (41) differential expressed genes from the list were significantly down-regulated, and seven (7) were statistically up-regulated. Pathway analysis showed that ABEE may have caused apoptosis of MCF-7 cells through extrinsic and or intrinsic apoptotic pathways. The analysis also suggested that activation of p53 could be the first step towards apoptotic elimination of the cancer cells upon treatment of ABEE. It is noted that the genes studied in this work were limited only to the array used and as such it would be interesting to look further into the effects on other genes.

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