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ORIGINAL ARTICLE

Tualang Honey Promotes Apoptosis of the A549 Lung Adenocarcinoma Cell Line via Modulation of PI3K/AKT Signaling Pathway-Related Proteins

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ABSTRAK

Isyarat fosfatidilinositol 3-kinases (PI3Ks)/protein kinase B (AKT) sering diekspresikan secara berlebihan dalam adenokarsinoma paru-paru dan dikaitkan dengan karsinogenesis melalui proliferasi sel, dan penyahaktifan apoptosis; dan ia juga meningkatkan rintangan terhadap ubat-ubatan kemoterapi. Walaupun madu tualang (TH) telah terbukti akan sifat antikansernya terhadap adenokarsinoma paru-paru, namun mekanisma asasnya masih belum dilaporkan. Oleh itu, kajian ini dilakukan untuk mengkaji kesan rangsangan apoptosis TH terhadap sel A549 adenokarsinoma paru-paru dengan menggunakan Array Apoptosis Manusia RayBio® dan analisis kuantitatif cecair spektrometri-jisim tandem kromatografi tanpa label (LC-MS/MS) untuk menjelaskan tentang modulasi protein hulu dan hiliran pada isyarat PI3K/AKT. Kesan penggalakan apoptosis oleh TH dikaitkan dengan (i) peningkatan kawalan protein pro-apoptosis (iaitu cytochrome c, histon H1.2, dan histon H1.4) dan protein penindas tumor (iaitu IGFBP-3 dan IGFBP-5), dan (ii) penurunan XIAP, sistem faktor pertumbuhan insulin (IGF) (iaitu IGF-1, IGF1R, IGFBP-1, IGFBP-2 dan IGFBP-4), HSP90AB1, YWHAQ, endoplasmin dan ITGB1. Kesannya juga dikaitkan dengan penindasan reseptor kinase tirosin, integrin, protein G, CHUK, RAC1, dan JAK. Oleh itu, TH boleh merangsang apoptosis pada sel A549 adenokarsinoma paru-paru melalui pengubahan protein berkaitan isyarat PI3K/AKT. Walau bagaimanapun, kajian lanjut perlu dibuktikan oleh penglibatan model haiwan untuk menyediakan model pemahaman yang lebih baik.

Kata kunci: apoptosis, A549 cells, fosfatidilinositol 3-kinases, madu tualang

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ABSTRACT

The phosphatidylinositol 3-kinases (PI3Ks)/protein kinase B (AKT) signaling pathway is frequently overexpressed in lung adenocarcinoma and associated with carcinogenesis through cell proliferation, and apoptosis deactivation; and it also enhances chemotherapeutic drugs resistance. Tualang honey (TH) has proven anticancer property on lung adenocarcinoma. However, the underlying mechanisms remain unreported. Hence, this study investigates the apoptosis-inducing effect of TH on A549 lung adenocarcinoma cell line using RayBio® Human Apoptosis Array and label-free quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to elucidate the modulation of upstream and downstream proteins of PI3K/AKT signaling pathway. The apoptosis-promoting effects of TH were associated with (i) the upregulation of pro-apoptotic proteins (i.e. cytochrome c, histone H1.2, and histone H1.4) and tumor suppressor proteins (i.e. IGFBP-3 and IGFBP-5), and (ii) downregulation of XIAP, insulin-growth factor (IGF) system (i.e. IGF-1, IGF1R, IGFBP-1, IGFBP-2, and IGFBP-4), HSP90AB1, YWHAQ, endoplasmin, and ITGB1. The effects were also linked with the suppression of receptor tyrosine kinase, integrins, G proteins, CHUK, RAC1, and JAK. Thus, TH may promote apoptosis of A549 lung adenocarcinoma cell line through alteration of the PI3K/AKT signaling pathway-related proteins. However, further studies involving animal models to provide a better model of understanding would prove necessary.

Keywords: apoptosis, A549 cells, tualang honey, phosphatidylinositol 3-kinases

INTRODUCTION

The most frequent subtype of non-small cell lung cancer (NSCLC) is lung adenocarcinoma, accounting for 40-45% of overall cases. It is frequently discovered at later stages when disseminated metastatic cancers have spread throughout the body. The resistance of lung adenocarcinoma toward standard radio- and chemotherapies poses a significant obstacle to therapeutic efficacy (Zagryazhskaya et al. 2015). Lung adenocarcinoma carries multiple somatic mutations and aberration of multiple signaling pathways (The Cancer Genome Atlas Research Network 2014). The epidermal growth factor receptor (EGFR) mutation is highly expressed in lung adenocarcinoma, accounting for 22% of total cases (Sholl et al. 2015). The activation of EGFR is upstream of phosphatidylinositol 3-kinases (PI3Ks)/ protein kinase B (AKT) signaling pathway and is often implicated in lung adenocarcinoma development and progression (The Cancer Genome Atlas Research Network 2014).

PI3Ks are made up of a family of lipid kinases. Their activation is stimulated

by multiple signals, such as growth factors (GFs), cytokines, integrins and other environmental cues that induce the production of phosphatidylinositol (3,4)-bis-phosphate (PIP2) and phosphatidylinositol (3,4,5)-tris-phosphate (PIP3). PIP3 interacts with AKT in the plasma membrane, causing the partial activation of AKT through threonine 308 phosphorylation by 3-phosphoinositide-dependent protein kinase 1 (PDK1). Following this, phosphorylation of the serine 473 in the C-terminal triggers the complete AKT activation (Hemmings & Restuccia 2012). The PI3K/AKT signaling pathway activation modulates numerous cellular functions in carcinogenesis including proliferation, survival, angiogenesis, invasion, and metastasis. Therefore, this pathway is a promising target for new anticancer therapies in lung adenocarcinoma as it often offers possible effects on sensitivity and resistance to conventional oncotherapy (Tan 2020). In the present study, we proposed the utilisation of honey as the new anticancer therapy targeting the PI3K/AKT.

Since ancient times, honey, has been widely used for various medicinal purposes. It was either eaten directly from the jar or mixed with water (Amran et al. 2020). Honey contains mixtures of supersaturated sugars, water, amino acids, polyphenols, vitamins, organic acids, minerals, pigments, waxes, and pollen grains (Machado De-Melo et al. 2018; Solayman et al. 2016). Despite the fact that the contents of honey vary depending on the nectar origin, geographical origin, entomological sources, environment, and processing factor (Alvarez-Suarez et al. 2010), the presence of polyphenols compounds have scientifically proven to be the main contributory factor for its anticancer properties (Mandal & Jaganathan 2009). Its polyphenol compounds such as caffeic acid, gallic acid, quercetin, apigenin, galangin, acacetin, pinocembrin, and kaempferol have been reported as promising pharmacological agents in treating cancer (Mandal & Jaganathan 2009; Ramon & Guillena 2020). Studies showed that the organic compounds such as sugar, organics acids, and amino acids in honey can act as a natural deep eutectic solvent and have a high ability to dissolve polyphenols (Dai et al. 2020; Funari et al. 2019). These unique characteristics prove that honey as a whole is held accountable for its anticancer effects and is also relatively safe to consume compared to individual compounds or extracts (Ahmed & Othman 2013). A recent study by Amran and colleagues reported that Tualang honey (TH) promotes antiproliferative activity and apoptosis in lung adenocarcinoma cell lines via extrinsic and intrinsic apoptosis pathways (Amran et al. 2020). However, the plausible explanation of its mechanism of inducing apoptosis is not fully understood. To our knowledge, this is the first study to investigate the effects of TH on modulating upstream and downstream proteins of PI3K/AKT signaling pathway in promoting apoptosis on A549 lung adenocarcinoma cell line.

MATERIALS AND METHODS

Sample Preparation

Tualang honey (TH) was acquired from the Malaysian Ministry of Agriculture and Agro-Based Industry's Federal Agricultural Marketing Authority (FAMA) (product code number: 110013). It was collected from Padang Terap in Kedah, Malaysia (6°15'N, 100°40'E). The water and sugar content of TH were standardised by FAMA at 21% and 73.5%, respectively.

A549 lung adenocarcinoma cell line (ATCC® No. CCL-185™, passage number 03-10) was procured from American Type Cell Culture (ATCC) (ATCC, Virginia, USA). The A549 cell line was propagated in RPMI-1640 (Biowest, Nuaille, France) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin (Biowest, Nuaille, France). In addition, a suitable cell culture practice such as a minimum passage number and routine cell line monitoring, including morphological appearance, proliferation ability, and protein expression, was implemented to maintain a stable phenotype and consistent cell performance (Geraghty et al. 2014).

A549 cells (1 x 10^5 cells/mL) were seeded in the T-75 flask until they reached 80-90% confluency. Cells were treated with 3.1% (v/v) concentration of TH for 24 h. The concentration was determined according to our previous 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay that showed inhibitory concentration at which 50% of the growth of the TH-treated cells was inhibited compared to the untreated cells (IC_{50}) (Amran et al. 2020). After 24 hours of incubation with TH, the medium was then discarded, and the cells were rinsed with cold phosphate-buffered saline (PBS). The cells were gently scraped, collected, and centrifuged at 1,000 g for 10 minutes at 4°C to obtain a cell pellet. The preparation of protein extraction and peptide from the cell pellet was carried out according to the manufacturer's protocol of RayBio® Human Apoptosis Antibody Array G-Series (RayBiotech, Inc., Peachtree Corners, GA, USA) and Pierce™ Mass Spec Sample Prep Kit for Cultured Cells (Thermo Scientific, Rockford, IL USA), respectively. The Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) was used to determine protein concentration. Experiments were carried out in three biological replicates. Cells without TH (untreated) representing as control.

Apoptosis Proteome Profiler Analysis

In determining the probable mechanism of action by which TH induced apoptosis, RayBio® Human Apoptosis Antibody Array G-Series (RayBiotech, Inc., Peachtree Corners, GA, USA) was used to investigate the apoptosis-related proteins, as previously described (Amran et al. 2020). A549 cell line was treated with 3.1% (v/v) of TH. According to the manufacturer's protocol of RayBio® Human Apoptosis Antibody Array G-Series, a total of 500 µg/mL of protein was extracted and then incubated with the human apoptosis array overnight. The Agilent G2565CA Microarray Scanner System

(Agilent Technologies, Santa Clara, CA, USA) in the Cy3 channel was used to scan the apoptosis array slide. The spot signal intensity was analysed using Image Studio™ Lite software (ver. 5.2), whilst background corrections and normalisation were carried out using the RayBio® Analysis Tool.

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) Analysis

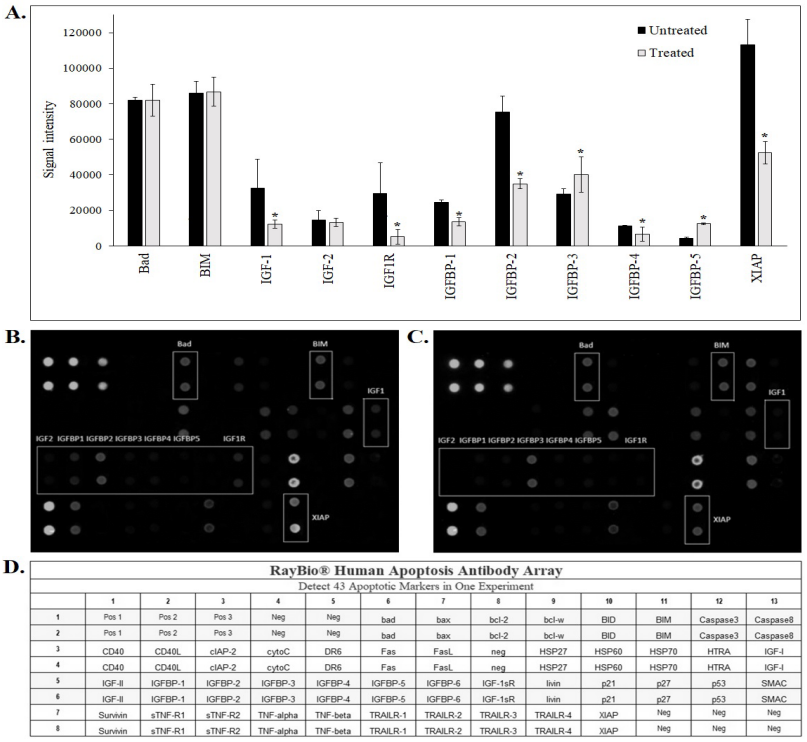
Waters® nanoACQUITY UPLC® system (Waters, Millford, MA, USA) was used to perform liquid chromatography analysis of digested peptides. Concentrated and desalted peptides (1 mg/mL) were loaded in Symmetry C18, 180 x 20 mm^2 trapping column and followed by separation on analytical column BEH130 C18, 100 µm x 100 mm. Peptides were eluted in the elution mobile phase with the following gradients: 5-40% in 91 minutes; 95% in 2 minutes; 95% in 6 minutes; and equilibrated back to 5% in 2 minutes for a total of 20 minutes at a flow rate of 300 nL min^{-1}. To ensure the sample ran continuously, two blanks were injected consecutively between each triplicate set of samples.

The quadrupole-time of flight (Q-TOF) Synapt G2 HDMS mass spectrometer (Waters, Millford, MA, USA) was used to analyse the isolated peptides utilising a positive ion mode electrospray ionisation (ESI). The mass spectrometer was calibrated by infusing 500 fmol/µL of [Glu-1]-fibrinopeptide through the reference sprayer of the NanoLockSpray source. The precursor ions (MS data) were assembled at a continuous rate of 6 eV. In contrast, the fragmentation ions (MSE data) were collected by ramping the voltage from 15 eV to 40 eV. A series of MS and MSE data were obtained every 4 s with a scan range from 50 to 1990 m/z.

Protein Identification and Label-free Quantification

ProteinLynx Global Server (PLGS) software version 2.4 from (Waters, Millford, MA, USA) was used to analyse continuum LC-MS/MS data. The search parameters were set up as follows: 10 ppm for the precursor tolerance, 20 ppm for fragment ion mass tolerances, carbamidomethylation of cysteine was assigned as a fixed modification, the deamidation of asparagine and glutamine and the oxidation of methionine were allocated as the variable modifications, 1000 counts intensity threshold, ≥2 peptides matched, ≥3 product ion per peptide, ≥7 ion matches per protein, low energy threshold of 100 counts, elevated energy threshold of 40 counts and 4% false-positive rate (FDR) with one missed cleavage. The UniProt Knowledgebase (https://www.uniprot.org) was used to search the generated MS/MS spectra from the software against *Homo sapiens* protein database. Three biological replicates for each treatment were injected thrice, then followed by blank and digested protein standard, consecutively after each injection of sample.

The Waters® Protein Expression Analyses Software, which is part of the PLGS package, was used to perform relative quantification in protein



D.

RayBio® Human Apoptosis Antibody Array

Detect 43 Apoptotic Markers in One Experiment

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	Pos 1	Pos 2	Pos 3	Neg	Neg	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	Caspase8
2	Pos 1	Pos 2	Pos 3	Neg	Neg	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	Caspase8
3	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	neg	HSP27	HSP60	HSP70	HTRA	IGF-I
4	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL	neg	HSP27	HSP60	HSP70	HTRA	IGF-I
5	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC
6	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC
7	Survivin	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Neg	Neg	Neg
8	Survivin	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Neg	Neg	Neg

Figure 1: Apoptosis proteome profiler analysis of A549 cells treated with TH for 24 h. (A) Quantitative analysis between untreated and treated cells, with the data expressed as the mean signal intensity ±SD (n=3). Expression of apoptotic proteins in (B) untreated and (C) TH-treated cells. (D) The exact protein name of each dot in the array. *P<0.05 compared to untreated cells.

abundance using MS ion intensities. The PLGS score of identified proteins was calculated using the Monte-Carlo algorithm, with the highest score indicating the most confidence in protein identification (Wright et al. 2009). The intensity of the variation coefficient (CV) of the three highest intense peptides was kept constant at 10% to ensure the system's reproducibility.

Bioinformatic Analysis

The list of identified proteins was submitted into Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (https://www.genome.jp/kegg/mapper/search.html) to analyse the representative pathway involved in the cancer-associated proteins and the pathway map was generated for visualisation purposes.

Statistical Analysis

Statistical analysis for human apoptosis proteome profile array was performed using the IBM SPSS, version 24.0 software (IBM Corp., Armonk, NY, USA). Data from three individual experiments were presented as means standard deviation (SD) (n=3, for each experiment). Independent

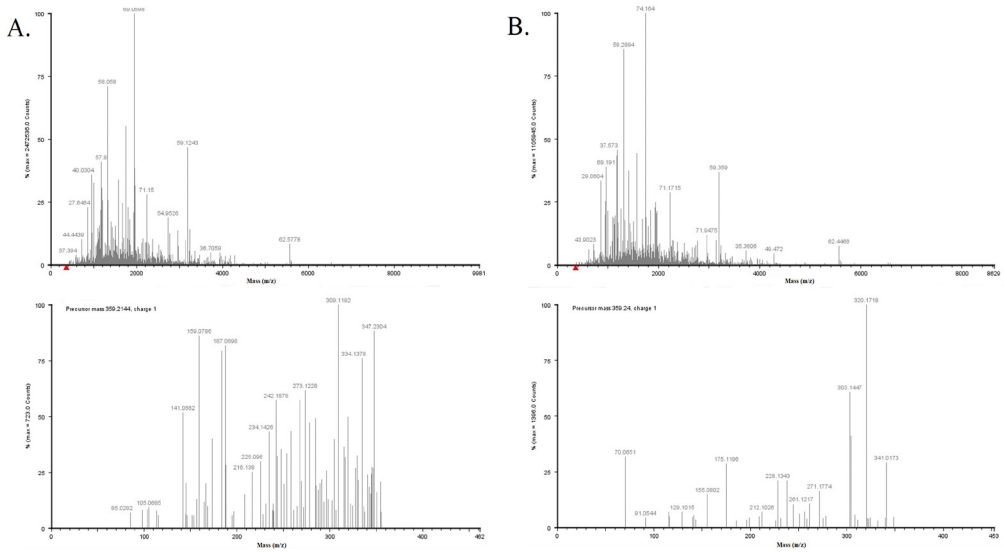


Figure 2: LC-MS/MS analysis for ion mass spectra of control (A) and treated (B) A549 cells after TH treatment for 24 hours.

Student's t-test was applied to compare treated and untreated samples. A value of $P<0.05$ was statistically significant.

RESULTS

Apoptosis Proteome Profiler Analysis

Profiling of key proteins involved in PI3K/AKT pathway-modulated apoptosis was carried out to determine the detailed mechanisms responsible for TH-induced apoptosis in A549 cells. As shown in Figure 1A, insulin-like growth factor (IGF) protein-ligand 1 (IGF-1), IGF receptor (IGF1R), three IGF binding proteins (IGFBP-1, IGFBP-2, and IGFBP-4), and X-chromosome-linked inhibitor of apoptosis (XIAP) were significantly ($P<0.05$) downregulated after 24 hours of treatment. In contrast, the expression of IGFBP-2 and IGFBP-5 were significantly ($P<0.05$) upregulated. At the same time, the expression level of Bad, BIM, and IGF-2 did not show any changes upon 24 h of treatment compared to the untreated cells.

Differentially Expressed Proteins in A549 Cells in Response to TH Treatment

In this study, label-free proteomic quantification of TH-treated A549 cells identified pertinent proteins in lung carcinogenesis, including proteins of crucial pathway mediators (Figure 2). Overall, a total of 1069 distinct proteins were quantified, and 133 of these proteins were revealed to be differentially expressed when the A549 cells were treated with 3.1% (v/v) TH for 24 hours. Among the differentially expressed proteins, 57 proteins were upregulated and 76 proteins were downregulated. However, 346 and 185 proteins were

Table 1: A summary of the total proteins identified by quantitative label-free LC-MS/MS

Protein expression	No. of protein	%
Downregulate proteins	76	7.1
Up-regulate proteins	57	5.3
Proteins unique to treated cells	185	17.3
Proteins unique to control cells	346	32.4

uniquely expressed in the control and TH-treated cells, respectively (Table 1). A total of 31 proteins were listed to be associated with the cancer pathway (Table 2) and 17 distinct proteins were found to be involved in the PI3K/AKT signaling pathway (Figure 3). These proteins such as EGFR, integrin alpha 3 (ITGA3), integrin alpha 6 (ITGA6), cyclic AMP-dependent transcription factor ATF 6 beta (ATF6B), guanine nucleotide-binding protein subunit beta 1 and beta 4 (GNB1 and GNB4), guanine nucleotide-binding protein subunit gamma 12 (GNG12), inhibitor of nuclear factor-kappa B kinase subunit alpha (CHUK), ras-related C3 botulinum toxin substrate 1 (RAC1), tyrosine-protein kinase JAK1 (JAK1), 40S ribosomal protein S6 (RPS6), and vascular endothelial growth factor receptor 1 (FLT1) were found to be suppressed by TH treatment. In comparison, the protein expression of heat shock protein HSP 90 beta (HSP90AB1), 14-3-3 protein theta (YWHAQ), endoplasmin (HSP90B1), and integrin beta 1 (ITGB1) were downregulated. The expression of apoptotic proteins such as cytochrome c, histone H1.2, and histone H1.4 was upregulated.

DISCUSSION

Honey has been long used as a traditional medicine additive and scientifically proven to have anticancer properties. Tualang Honey (TH) has been reported to show anticancer effects on various types of cancers including breast, oral, leukemia, cervical and lung cancer. Researchers discovered that TH displays anticancer effects by inhibiting proliferation, apoptosis induction, and cell cycle arrest via modulations of targeted proteins such as p21, p53, Bcl-2, caspases activation, Apfa-1, p53, IFN-γ, IFNGR1, TNF-α, COX-2, FADD, Bid, Bax, cytochrome c, SMAC (second mitochondria-derived activator of caspase), and Fas (Ahmed et al. 2017; Ahmed & Othman 2017; Amran et al. 2020; Fauzi et al. 2011; Fauzi & Yaacob 2016; Ghashm et al. 2010; Kadir et al. 2013; Yaacob et al. 2013). Despite several anecdotal claims postulating its anticancer role on various types of cancer, empirical studies on lung adenocarcinoma with the plausible explanation of its mechanism of inducing apoptosis, remain unreported. In this study, we utilised a quantitative proteomic method to elucidate the involvement of PI3K/AKT signaling pathway in apoptosis induced by TH.

The differentially expressed proteins were identified to be predominantly related to lung adenocarcinoma. They play a crucial function in carcinogenesis through sustaining proliferation, evading apoptosis, inducing angiogenesis, activating tumor invasion, and migration. These results were in agreement with previous

Table 2: List of differentially expressed proteins associated with cancer-related signalling pathway as identified by LCMS/MS.

ID[1]	Description	Gene name	PLGS score[2]	Peptide[3]	Log of ratio[4]	Unique to (untreated or treated)	Signalling pathway
P27348	14-3-3 protein theta	*YWHAQ*	785.611	3	-0.39±0.37	-	PI3K/AKT
P62753	40S ribosomal protein S6	*RPS6*	219.773	8	-	Untreated	PI3K/AKT
P35221	Catenin alpha 1	*CTNNA1*	132.382	5	-	Untreated	Adherens junction
P35222	Catenin beta 1	*CTNNB1*	63.2014	28	-	Untreated	Adherens junction
P60953	Cell division control protein 42 homolog	*CDC42*	190.644	7	-	Untreated	MAPK
Q99941	Cyclic AMP dependent transcription factor ATF 6 beta	*ATF6B*	58.0977	9	-	Untreated	PI3K/AKT
P99999	Cytochrome c	*CYCS*	2132.78	9	0.39±0.46	-	Apoptosis
P14625	Endoplasmin	*HSP90B1*	3032.92	9	-0.13±0.07	-	PI3K/AKT
Q99814	Endothelial PAS domain containing protein 1	*EPAS1*	63.2327	13	-	Untreated	HIF-1
P00533	Epidermal growth factor receptor	*EGFR*	81.2064	5	-	Untreated	PI3K/AKT
Q9UBI6	Guanine nucleotide binding protein G I G S G O subunit gamma 12	*GNG12*	738.565	30	-	Untreated	PI3K/AKT
P62873	Guanine nucleotide binding protein G I G S G T subunit beta 1	*GNB1*	68.1593	26	-	Untreated	PI3K/AKT
P63092	Guanine nucleotide binding protein G s subunit alpha isoforms short	*GNAS*	369.213	3	-0.76±0.88	-	cAMP
Q9HAV0	Guanine nucleotide binding protein subunit beta 4	*GNB4*	40.4501	25	-	Untreated	PI3K/AKT
P08238	Heat shock protein HSP 90 beta	HSP90AB1	2509.6	6	-0.32±0.19	-	PI3K/AKT
P09601	Heme oxygenase 1	HMOX1	5197.4	8	-1.68±0.75	-	HIF-1

ID[1]	Description	Gene name	PLGS score[2]	Peptide[3]	Log of ratio[4]	Unique to (untreated or treated)	Signalling pathway
P16403	Histone H1.2	*HIST1H1C*	5144.56	3	0.88±0.10	-	Apoptosis
P10412	Histone H1.4	*HIST1H1E*	5090.53	16	0.34±0.10	-	Apoptosis
O15111	Inhibitor of nuclear factor kappa B kinase subunit alpha	*CHUK*	48.0157	10	-	Untreated	PI3K/AKT
P26006	Integrin alpha 3	*ITGA3*	61.647	35	-	Untreated	PI3K/AKT
P23229	Integrin alpha 6	*ITGA6*	123.334	3	-	Untreated	PI3K/AKT
P05556	Integrin beta 1	*ITGB1*	802.401	6	-0.23±0.08	-	PI3K/AKT
P14923	Junction plakoglobin	*JUP*	60.6575	4	-	Untreated	Cancer
Q13772	Nuclear receptor coactivator 4	*NCOA4*	42.106	10	-	Untreated	MAPK
Q06710	Paired box protein Pax 8	*PAX8*	58.5069	6	-	Untreated	PPAR
P63000	Ras related C3 botulinum toxin substrate 1	*RAC1*	250.679	6	-	Untreated	PI3K/AKT
P11233	Ras related protein Ral A	*RALA*	337.607	7	-	Untreated	RAP1
P63208	S phase kinase associated protein 1	*SKP1*	668.077	5	-	Untreated	Cell cycle
P30153	Serine/ threonine protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	*PPP2R1A*	98.1105	10	-	Treated	PI3K/AKT
Q14765	Signal transducer and activator of transcription 4	*STAT4*	68.0331	7	-	Untreated	JAK/STAT
P06753	Tropomyosin alpha 3 chain	*TPM3*	111.557	27	-	Untreated	MAPK
P23458	Tyrosine protein kinase JAK1	*JAK1*	47.1147	6	-	Untreated	PI3K/AKT
P17948	Vascular endothelial growth factor receptor 1	*FLT1*	49.8047	19	-	Untreated	PI3K/AKT

[1]Accession ID in the Uniprot database. [2]PLGS score is calculated by the Protein Lynx Global Server (PLGS 2.2.5). [3]peptides used for quantification. [4]log of ratio (untreated:TH-treated) with positive and negative values indicating up- and down-regulation of protein expression.

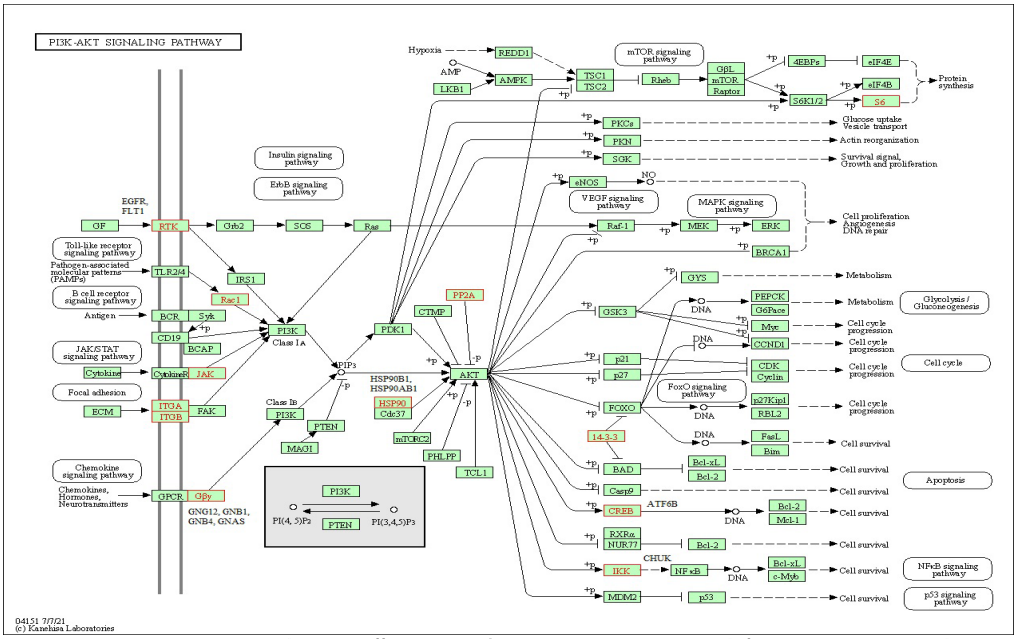


Figure 3: Systematic diagram illustrating the effect of TH on modulating upstream and downstream proteins of PI3K/AKT signaling pathway generated by KEGG

findings that showed proteins such as EGFR, guanine nucleotide-binding protein G (GNAS), cell division control protein 42 homolog, heme oxygenase 1, tropomyosin alpha 3 chain, S phase kinase-associated protein 1, catenin alpha 1, β-catenin beta (CTNNB1), YWHAQ, and HSP90AB1 were implicated in lung adenocarcinoma development and progression (Korrodi-Gregório et al. 2016; Qi et al. 2005; Sholl et al. 2015; Wang et al. 2016; Yang et al. 2017).

It was observed that TH deactivates PI3K/AKT signaling pathway through modulation of multiple upstream signaling proteins such as receptor tyrosine kinase (RTK), integrins, G proteins, Janus kinase, and toll-like receptor; and downstream PI3K/AKT-related proteins such as ATF6B, CHUK, YWHAQ, and RPS6 after 24 hours of TH treatment. As shown in Figure 3, suppression of RTK proteins such as EGFR and FLT1 contributes to inhibiting cancer cell proliferation and apoptosis induction. Downregulation of JAK1, a protein associated with Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway also contributes to the inhibition of A549 cells proliferation by deactivating PI3K. The interaction of HSP90 with the pro-survival kinase AKT protects AKT from being degraded by proteasomes, which helps to maintain the functional stability of PI3K/AKT signaling and cell survival (Redlak & Miller 2011). Downregulation of HSP90 by TH treatment contributes to apoptosis induction in A549 cells via AKT deactivation. The downstream activation of PI3K/AKT signaling pathway by downregulating or

suppressing the expression of proteins such as CHUK, ATF6B, YWHAQ, and cytochrome c may also contribute to the apoptosis in A549 cells.

Insulinlike growth factors (IGF) are peptides growth factors that play a crucial role in autocrine/ paracrine growth factors and endocrine hormones promoting cell development and survival. They are regulated by a family of six IGF binding proteins (IGFBPs), which can cause the inhibition or stimulation of IGF effects and prolong the IGF half-life in the circulation (Allard & Duan 2018). Furthermore, these IGFBPs serve as carriers for IGFs to the receptors on cell surfaces (Kostecká & Blahovec 2002). All six IGFBPs (IGPB-1 to -6) share an equal or greater binding affinity for IGFs compared to the IGF receptor (IGF1R) (Allard & Duan 2018).

Our results showed that TH treatment downregulated the expression of IGF-1, IGF1R, IGFBP-1, IGFBP-2, and IGFBP-4. These proteins are known to be overexpressed in lung adenocarcinoma, implicating tumor proliferation, survival, and aggressiveness (Fu et al. 2016; Shersher et al. 2011). Studies have shown that the inhibition of IGF-1 and IGF1R can induce the intrinsic apoptosis pathway by decreasing the level of anti-apoptotic Bcl-2 proteins, increasing caspase-3 activity, and modulating the activity of death-inducing receptors (Kooijman 2006; Zhang et al. 2018). Moreover, the activation of PI3K is triggered by IGF-1 binding to IGF1R and leads to the deactivation of apoptosis (Chen et al. 2014; Fernández et al. 2004). Our results also showed that TH treatment upregulated the expression of IGFBP-3 and IGFBP-5. Upregulation of these proteins implies tumor growth inhibition. IGFBP-3 is known to have a role as a potent apoptosis inducer in NSCLC cells through the suppression of IGF-mediated signaling pathway, while IGFBP-5 is a tumor suppressor (Lee et al. 2002; Rho et al. 2008).

Our results showed TH activated apoptosis in the A549 cells by upregulating the expression of cytochrome c, Histone H1.2, and Histone H1.4, while concomitantly downregulating the expression of XIAP. Deactivated XIAP can further activate downstream cytochrome c, leading to tumor cell apoptosis and directly suppresses PI3K/AKT signaling pathway (Hemmings & Restuccia 2012). Histone H1.2 and H1.4 are essential proteins for apoptosis induction. Histone H1.2 triggers the production of cytochrome c from mitochondria through activation of caspase-3 activation and histone H1.4 promotes apoptosis via chromatin condensation (Clausell et al. 2009; Ruiz-Vela & Korsmeyer 2007). These findings were substantiated by a report that indicated apoptosis induction of lung adenocarcinoma through chromatin condensation and activation of caspase-3 after being treated with TH (Amran et al. 2020). Therefore, downregulating these proteins expression may pose an attractive target to ensure apoptosis induction by TH in lung adenocarcinoma cells.

Mandal and Jaganathan (2009) have scientifically proven that the presence of polyphenol compounds is the most important factor in its contribution to anticancer properties. Tabasum

and Singh (2019) showed that fisetin downregulates the expression of EGFR and catenin beta 1 (β-catenin) in A549 cells. Another study demonstrated that catechin inhibits the A549 cell proliferation by inhibiting the expression of AKT phosphorylation (Sun et al. 2020). Vitexin induces apoptosis in A549 cells by reducing the level of PI3K, Akt and mTOR phosphorylation (Liu et al. 2019). We postulated that the active compounds in TH such as fisetin, catechin and vitexin might contribute to apoptosis induction on lung adenocarcinoma cells (Chew et al. 2018; Moniruzzaman et al. 2013).

Although these findings facilitate an underlying understanding of the involvement of identified proteins associated with PI3K/AKT signaling pathway in response to TH treatment, further validation experiments including *in vivo* investigations are merit consideration.

CONCLUSION

The present study demonstrates for the first time that TH modulates the upstream and downstream proteins PI3K/AKT signaling pathway in promoting apoptosis of A549 lung adenocarcinoma cell line. This was achieved by upregulating the expression of apoptotic proteins (cytochrome c, H1.2, and H1.4); downregulating the expression of an anti-apoptotic protein (XIAP), growth factors (IGF-1, IGF1R, IGFBP-1, IGFBP-2, IGFBP-4), heat shock proteins (HSP90AB1 and HSP90B1), and suppression of oncogenes (EGFR, FLT1, JAK1 and CHUK), integrins and G proteins.

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