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Anticancer activity of propolis flavonoid pinobanksin-3-acetate against human colon cancer *in vitro*

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Abstract

Pinobanksin-3-acetate (PB3A) is a natural flavonoid commonly present in propolis, honey, and many plants. In this study, we evaluate the anticancer activity of PB3A on human colon cancer *in vitro*, and to elucidate the mechanism of its activity. Cells were tested *in vitro* for cytotoxicity, apoptotic peaks and gene expression profiling after treatment with PB3A, in which conducted cell proliferation assay, Flow cytometric (FCM) method and Human Genome Gene chip Array U133_Plus_2 analysis, respectively. PB3A exerted a significant inhibitory effect on SW480 cell proliferation and in a time- and dose-dependent manner, and PB3A could significantly increase the apoptosis rate in a dose-dependent manner. The gene profiling analysis identified that PB3A intervention induced a wide range of changes in the gene expression profile of SW480 cells. The findings in this study suggested that PB3A demonstrated potential anticancer activity on colon cancer *in vitro*, and it could induce apoptosis; which could be attributed, in part, to its inhibition of proliferation and induction of apoptosis in cancer cells through up-regulation and down-regulation of multiple genes involved in cell apoptosis, cytokinetics, colorectal carcinogenesis, Wnt, and Calcium signaling.

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Introduction

Colorectal cancer (CRC) is one of the most common human malignancies, and is the fourth leading cause of cancer deaths. All CRC patients including those that are post-surgery and have a late stage diagnosis need to receive chemotherapy. Although current chemotherapy (three most commonly used drugs Irinotecan, oxaliplatin, capecitabine in combination with adjuvant 5-fluorouracil) prolongs survival in most patients, complete cure cannot be achieved. Hence, more potential therapeutic drugs need to be developed. Aberrant WNT pathway signaling is an early progression event in 90% of colorectal cancers (Wang *et al.,* 2005). CRC has two types, colon cancer and rectal cancer, in this research we mainly focused on colon cancers *in vitro* using the cell line SW480.

Propolis, a resinous substance collected by bees from various plants, has a pleasant aromatic odor and yellow green to dark brown color depending on source and age (Awale *et al*., 2005), a natural beehive product. Chemical studies showed the complex composition of propolis, with more bioactive constituents, including several phenolic compounds such as derivatives of hydroxycinnamic acids and flavonoids (Bankova *et al*., 2000). Propolis has been reported to possess various biological activities, such as antibacterial (Kujumgiev *et al.,* 1999; Kartal *et al.,* 2003), antiviral (Amoros *et al.,* 1994; Kujumgiev *et al.,* 1999), anti-oxidation (Isla *et al*., 2001; Shigenori Kumazawa *et al.,* 2004), anti-inflammatory (Khayyal *et al.,* 1993; Hu *et al.,* 2005), anticancer (Kimoto *et al.,* 2001; Sforcin, 2007), antifungal (Kujumgiev *et al.,* 1999; Murad *et al.,* 2002), neuroprotective properties (Inokuchi *et al.,* 2006), and immunological adjuvant action (Fischer *et al.,* 2007). Some of the biological activities might be attributed to the identified high content of flavonoids from its chemical constituents (Njintang *et al.,* 2012).

Flavonoids are polyphenolic compounds that are present in plants. They have been shown to possess a variety of biological activities at non-toxic concentrations in organisms. Flavonoids are not xenogenic to humans and have minimal or no toxicity (Tiakavkina *et al.,* 1996). Flavonoids in propolis show anti-tumor effects which results from inducing apoptosis in target cells. Induced apoptosis is one of the mechanisms used for several therapeutic agents shown in propolis by several related researchers. Caffeic acid phenethyl ester (CAPE), an active component of propolis, has many biological and pharmacological activities including an anticancer effect (Wang *et al.,* 2005). It has been demonstrated that Propolin C, another active component of propolis, inhibits the proliferation of human melanoma cells through inducing a cytotoxic effect and triggering apoptosis (Chen *et al.,* 2004). It has been reported that Artepillin C from propolis exhibits anti-tumor effects by the induction of apoptosis in human tumor cells *in vitro* (Tetsuo *et al.,* 1998).

PB3A (Fig. 1), a member of the flavonone class of flavonoids, is present in high concentrations in medicinal plants (e.g. Pine and Aspen), honey and propolis, and exhibits moderate antioxidant activity which was first reported in 1988 (Fang *et al.,* 1988; Shigenori Kumazawa *et al.,* 2004). In current study, we evaluated PB3A's potential anti-cancer activity against colon cancer cell line SW480. PB3A demonstrated a significant inhibitory effect on cell proliferation and induced cell apoptosis, and it showed a little enhancement effect on 5-FU cytotoxicity. These effects of PB3A on colon cancer cell *in vitro* are associated with induce alterations of gene expression patterns.

Materials and methods

Drugs and reagents

PB3A (mass fraction≥99%) was isolated from propolis and its isolation process was not given in this paper, 5-FU (5-fluorouracl) purchased from Shanghai biotechnology corporation, China, Dimethyl Sulphoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) purchased from Sigma-Aldrich, USA. RPMI-1640 medium was purchased from Hyclone. Annexin-V and PI double staining kit was purchased from Biobest Company, China. PB3A was isolated from propolis and prepared as 100 μg/μl and 5-FU was prepared as 10 μg/μl stock solutions in DMSO. Control flasks or plates contained

DMSO at an equivalent dilution to that in cultures containing PB3A and 5-FU.

Cell line and culture

Human CRC SW480 cell line was obtained from the Chinese academy of sciences Cell Bank (Shanghai, People's Republic of China). These cells were grown at 37℃ and in a 5% CO_2 atmosphere in the appropriate growth media (RPMI 1640 medium, pH 7.2-7.4) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 μg/ml)/streptomycin (100 U/ml) solutions. Cells were routinely sub-cultured using a trypsin/ethylenedinitrile tetra-acetic acid (EDTA) solution (obtained from Sigma).

Cell proliferation assay

The effect of PB3A and 5-FU on cell proliferation was measured using the MTT assay. The logarithmically growing SW480 cells (5×10^3 /well) were plated in sterile 96 well plates. After 24 h, these cells were incubated with media containing PB3A at various concentrations (150, 100, 50, 25, and 5 μg/ml), 5-FU as a positive control with various concentrations (50, 25, and 5 μg/ml) and PB3A+5-FU at respective concentrations; control wells were incubated with media containing 0.5% DMSO alone, for 24, 48, and 72 h. After the incubation period, 10 μl of 5 mg/ml MTT (pH 4.7) reagent was added per well and incubated for another 4 h at 37℃, the supernatant fluid was removed and 150 μl DMSO was added per well and shaken for 10 min. The absorbance at 570 nm was measured with a microplate reader (Bio-Tek, Winooski, VT), using wells without cells as blanks. All experiments were performed in triplicate. The inhibition of cells by PB3A was calculated as a percentage value compared to control. The absorbance (A) was measured at 570 nm and the inhibition ratio (%) was calculated using the following equation:

Inhibitory ratio (%) = [(A Control- A Treated)/ A Control] ×100%.

IC_{50} was taken as the concentration that caused 50% inhibition of cell viabilities and calculated by the Logit method (Fazal *et al.,* 2005; Shi *et al.,* 2008).

Annexin V/PI double staining assay

Apoptosis-mediated cell death of tumor cell SW480 was examined using a double staining method with the FITC-labeled Annexin V/PI Apoptosis Detection kit (Biobest, China) according to the manufacturer's instructions. FCM analysis was performed 10～15 min after supravital staining. Data acquisition and analysis were performed in a Becton Dickinson FACS Calibur flow cytometer using Cell Quest software (BD Biosciences, Franklin Lakes, and NJ). The left lower section of fluorocytogram (An-, PI-) represents the normal cells, the right lower section represents early and median apoptosis cells, the right upper section represents late apoptosis cells (Jalving *et al.,* 2006; TLiu *et al.,* 2007).

Microarray analysis

Human colon cancer SW480 cells were treated with 100 μg/ml of PB3A for 24 h. Total RNA was extracted from the treated and untreated cells using the Trizol one-step method. The gene expression profiles were analyzed by Human Genome Gene Chip Array U133 Plus_2 (HG-U133_Plus_2) and the differential expression of genes was compared between the control groups. The PB3A intervention group cells were classified according to their function using the Molecule Annotation System 3.0 (MAS 3.0). Scanned photography of microarray chips were digitalized and further analyzed by photo analysis in special facilities. Fold changes were calculated and expressed relative to untreated control sample for each pair of spots: the results were confirmed by duplicate spots of cRNA. Differentially expressed genes between the control and treated group were classified with Gene-Pathway Network was made by using MAS 3.0.

Statistics

Statistical analysis was performed with SPSS 17.0 general linear model univariate ANOVA and regression analysis where all experimental results were presented as mean±SD and 50%inhibited

concentration (IC_{50}). The p values $p < 0.05$ and $p < 0.01$ were considered to indicate significance.

Results

Cytotoxic activity of PB3A against human colon cancer cell line

PB3A exerted a significant inhibitory effect on SW480 cell proliferation in a time- and dose-dependent manner (Fig. 2A). The PB3A and 5-FU combination group result indicated a little enhancement effect on 5-FU cytotoxicity (Fig. 2B). The PB3A IC_{50} value for SW480 at 24, 48 and 72 h was 163.61 μg/ml, 40.72 μg/ml and 45.56 μg/ml, respectively. In this study, we demonstrated that PB3A is one of the active components from propolis that possesses a cytotoxic activity to colon cancer *in vitro*.

Table 1. Genes in the PB3A-treated group down-regulated 10-fold relative to the control group.

Probe Set ID	Ratio	Gene Symbol	Chromosomal Location	RefSeq Transcript ID
229784_at	0.0337	MGC16121	chrXq26.3	NR_024607..
209774_x_at	0.0477	CXCL2	chr4q21	NM_002089
204470_at	0.0652	CXCL1	chr4q21	NM_001511
228962_at	0.0679	PDE4D	chr5q12	NM_001104631
209285_s_at	0.0721	C3orf63	chr3p14.3	NM_001112736
213425_at	0.0815	WNT5A	chr3p21-p14	AI968085
211548_s_at	0.0841	HPGD	chr4q34-q35	J05594
226426_at	0.0861	ADNP	chr20q13.13	BG149849
244023_at	0.0876	SYK	chr9q22	AW467357
203914_x_at	0.0897	HPGD	chr4q34-q35	NM_000860
227578_at	0.0926	LOC100128191	chr12q23.1	H28597
225728_at	0.0935	SORBS2	chr4q35.1	AI659533
212023_s_at	0.0991	MKI67	chr10q25-qter	AU147044

Table 2. Genes in the PB3A-treated group up-regulated 10-fold relative to the control group.

Probe Set ID	Ratio	Gene Symbol	Chromosomal Location	RefSeq Transcript ID
213418_at	240.72	HSPA6	chr1q23	NM_002155
204475_at	77.23	MMP1	chr11q22.3	NM_001145938
204472_at	47.01	GEM	chr8q13-q21	NM_005261
206924_at	27.81	IL11	chr19q13.3-q13.4	NM_000641
203665_at	24.38	HMOX1	chr22q12|22q13.1	NM_002133
1557636_a_at	20.49	C7orf57	chr7p12.3	NM_001100159
202388_at	19.43	RGS2	chr1q31	NM_002923
213139_at	17.77	SNAI2	chr8q11	NM_003068
1554333_at	16.84	DNAJA4	chr15q25.1	NM_001130182
205352_at	13.25	SERPINI1	chr3q26.1	NM_005025
223710_at	12.86	CCL26	chr7q11.23	AF096296
200800_s_at	12.56	HSPA1A	chr6p21.3	NM_005345
210090_at	11.87	ARC	chr8q24.3	AF193421
225955_at	11.45	LOC653506	chr17q25.3	BG231494
205239_at	11.33	AREG	chr4q13-q21	NM_001657
202843_at	11.13	DNAJB9	chr7q31|14q24.2-q24.3	NM_012328
39248_at	10.83	AQP3	chr9p13	N74607
230643_at	10.74	WNT9A	chr1q42	BE220265
209457_at	10.09	DUSP5	chr10q25	U16996
209189_at	10.04	FOS	chr14q24.3	BC004490

PB3A induces apoptosis in SW480

After SW480 cells were exposed to PB3A (0, 50 and 100 μg/ml) and positive control 5-FU (50 μg/ml) for 24 h. Annexin-V and PI double-staining FCM analysis showed that early and late apoptosis rates for PB3A-treated SW480 cells were 7.20± 0.42% and 3.40 ± 0.70%, 32.50 ± 1.41% and 9.55 ± 1.34%, 49.60 ± 1.41% and 24.10 ± 0.98%. Early and late apoptosis rates after 5-FU treatment were 14.50 ± 0.71% and 3.80 ± 0.85% respectively (Fig. 3). FCM analysis showed that PB3A could significantly increase the apoptotic rate, suggesting a dose-dependent manner. Greater apoptotic and necrotic rates were observed when along with the increase of drug concentration; the induction of apoptotic rates were showed dose-dependent manner.

Table 3. The pathways associated with differentially expressed genes between the PB3A treated group and control group of SW480 cells.

Pathway	Up-regulated genes	Down-regulated genes	p-Value
MAPK	HSPA6, HSPA1A, DUSP1, NR4A1, HSPA1B, GADD45A, GADD45G, DUSP2, MAP3K14, ELK4, GADD45B, FOS, DDIT3, DUSP5, NR4A1, DUSP10, STK4	PDGFB, RASGRP3, NF1, FGF3, PDGFB, FGF20, NTRK2, SOS1	8.90238E-13
Cytokine-cytokine receptor interaction	IL6R, TNFSF9, IL11, TNFRSF10B, VEGFA, CCL26, KITLG, TSLP	TNFSF10, CXCL1, TNFRSF11B, CCL20, CXCL6, IL10, CXCL3, CXCL2, PDGFB, EDAR, TNFRSF19	9.64266E-10
p53	SERPINE1, GADD45A, GADD45G, TNFRSF10B	GTSE1	1.35648E-08
Hedgehog	BMP6, WNT9A	GLI3, WNT5A, GLI2, PTCH1, WNT6, GLI3, WNT3	5.02235E-07
Cell cycle	GADD45A, GADD45G, GADD45B	BUB1B, CDC7, RBL1, SKP2, ATM, LOC651610, CCNB1	2.44588E-06
Wnt	FOSL1, FZD10, WNT9A	ROCK2, WNT5A, FZD2, TBL1XR1, WNT6, AXIN2, WNT3, VANGL1	3.35842E-06
Colorectal cancer	FOS, FZD10	BIRC5, IGF1R, FZD2, AXIN2, SOS1	8.44232E-05
TGF-β	BMP6, SMAD6, ID1	ROCK2, SMAD7, RBL1, SMURF2	0.000105506
Apoptosis	MAP3K14, CASP7, TNFRSF10B	TNFSF10, BIRC3, ATM	0.000121801
Calcium	HRH1, SPHK1	ITPKB, ADCY9, ATP2A2, GNAQ, GNAS, PHKA1	0.002174104

Gene Chip scanning image and cell gene expression spectrum

In HG-U133_Plus_2 we have screened 54613 oligonucleotides from SW480 cells. The comparative analysis of the signal intensity between the experimental group and the control group showed that every spot represents one oligonucleotide two kinds of cells hybridization signal, at last makes for scatter plot (Fig. 4). 4912 differentially expressed genes were identified in which the gene expression level was increased or decreased more than 2-fold in cells treated with 100 μg/ml of PB3A compared with control cells cultured under identical condition without drug treatment. 1671 (34%) genes were up-regulated and 3241 (66%) genes were down-regulated. 551 genes showed more than 3-fold up-regulation and 848 genes showed 3-fold down-regulation. 13 genes showed more than 10-fold down-regulation and 20 genes showed 10-fold up-regulation (Table 1 and 2). In table 1 and 2, the gene symbol is referred to as its official name, and the probe sets are specific for particular sequences.

PB3A induced differential expressed genes Gene-Pathway Network

Gene-Pathway Networks were created using the KEGG database (**Fig. 5**). Data analysis showed that differentially expressed genes function involves 129

pathways. The key pathways among these networks involve Hedgehog, Wnt, TGF-β, cell cycle controlling, MAPK, colorectal carcinogenesis, p53, cell apoptosis, Calcium, and cytokinetics signaling (Fig. 5, Table 3). In table 3, differentially expressed gene pathways are classified as official classification, the p-value used in the algorithm is the hyper geometric distribution, when the mean p-value less than 0.05 for the difference in the genes in this pathway significantly enriched. In which TNFRSF10B, SOS1, TNFSF10, WNT5A, WNT6, WNT3 and ATM etc genes are belonged to two or more than two kinds of signaling pathways.

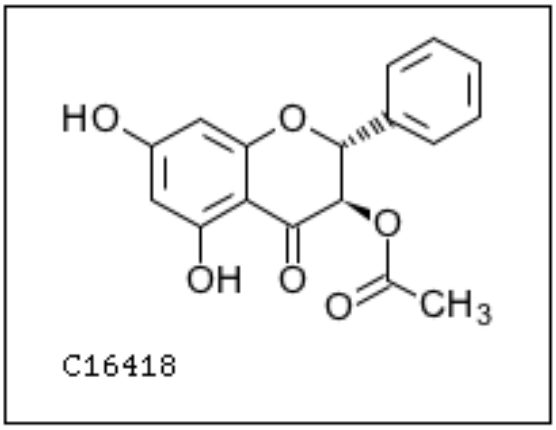

Fig. 1. Structures of PB3A.

Discussion

In our study, we found that PB3A has strong anti-tumor effects and could be identified as a unique constituent of propolis. In the present research, we investigated the effect of PB3A on the proliferation and cell apoptosis of colon cancer cell SW480. Our data demonstrates that PB3A treatment is associated with a strong inhibition of proliferation in a dose- and time-dependent manner, along with induction of cell apoptosis in colon cancer *in vitro*.

The results of gene expression profiles were analyzed by HG-U133_Plus_2 showed that PB3A intervention induced a wide range of changes of the gene expression profile of SW480 cells. A total of 551 genes were up-regulated and 848 genes were down-regulated by more than 3-fold compared to the control group, in which 20 genes were up-regulated and 13 genes were down-regulated by more than 10-fold. The genes that were differentially regulated by more than 3-fold were closely correlated with many kinds of cell signal transduction or gene expression regulation pathways, which include cell cycle control, cell apoptosis, cytokinetics, colorectal carcinogenesis, p53, DNA polymerase, Wnt, and Calcium signaling. Some of these belong to important signaling pathways related with cell proliferation and apoptosis such as ROCK2, WNT5A, FZD2, TBL1XR1, WNT6, AXIN2, WNT3, VANGL1, FOSL1 and FZD10 in the Wnt signaling, TNFSF10, BIRC3, ATM, MAP3K14, CASP7 and TNFRSF10B in apoptosis signaling, and SERPINE1, GADD45A, GADD45G, GTSE1, GADD45B in the p53 signaling pathway.

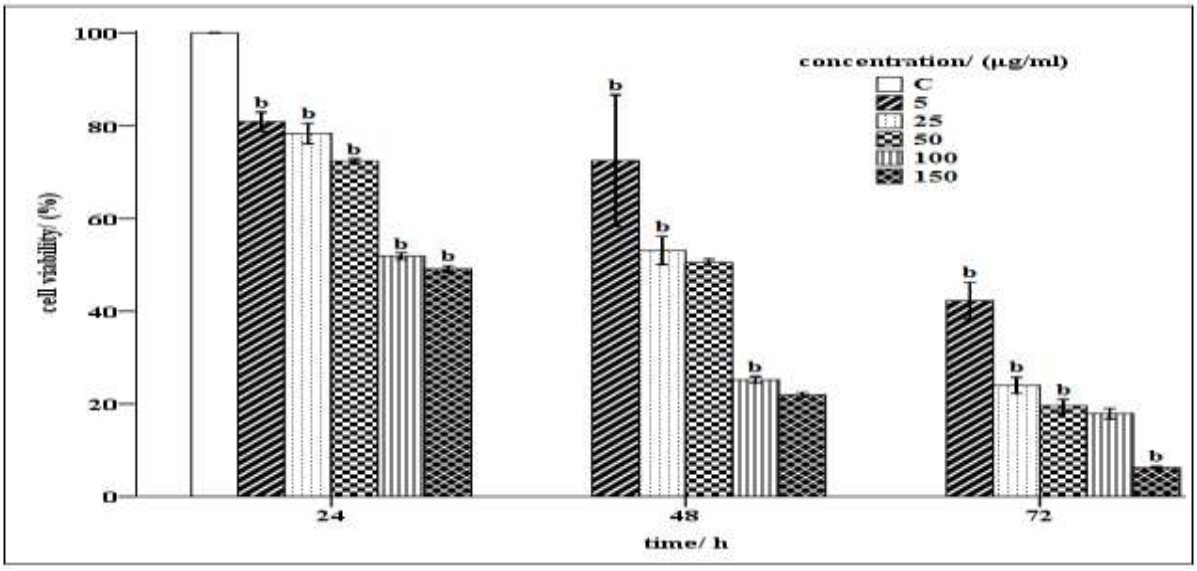

Fig. 2A.

From this study we found that PB3A is able to induce many alterations in gene expression patterns to show anticancer effects, e.g. the down-regulations of cytokine-cytokine receptor interaction factor genes CXCL2, CXCL1, and TNSF10. PB3A can also induce many alterations in some gene expression patterns to indicate drug resistance, e.g. the higher up-regulations of heat shock proteins HSPA6, HSPA1B and HSPA1B (Fant *et al.,* 2010; Varney *et al.,* 2011; Noonan *et al.,* 2008; Noonan *et al.,* 2008; Ashkenazi *et al.,* 2008).

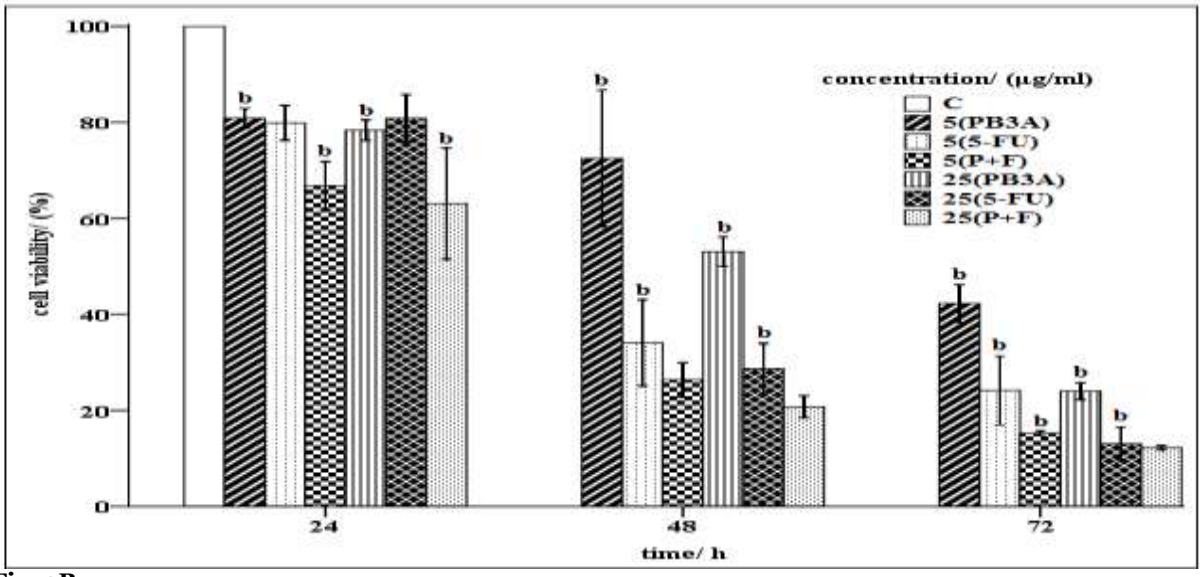

Fig. 2B.

Fig. 2. Effect of PB3A on the growth of colon cancer cells. Differences between the adjacent groups at the same time, $^{a}p<0.05$ and $^{b}p<0.01$, (results obtained from single experiment, $n=3$). (A) PB3A inhibits SW480 cell proliferation in a dose and time-dependent manner. (B) Synergistic inhibition effects of the PB3A co-treated with 5-FU on the SW480 cell proliferation.

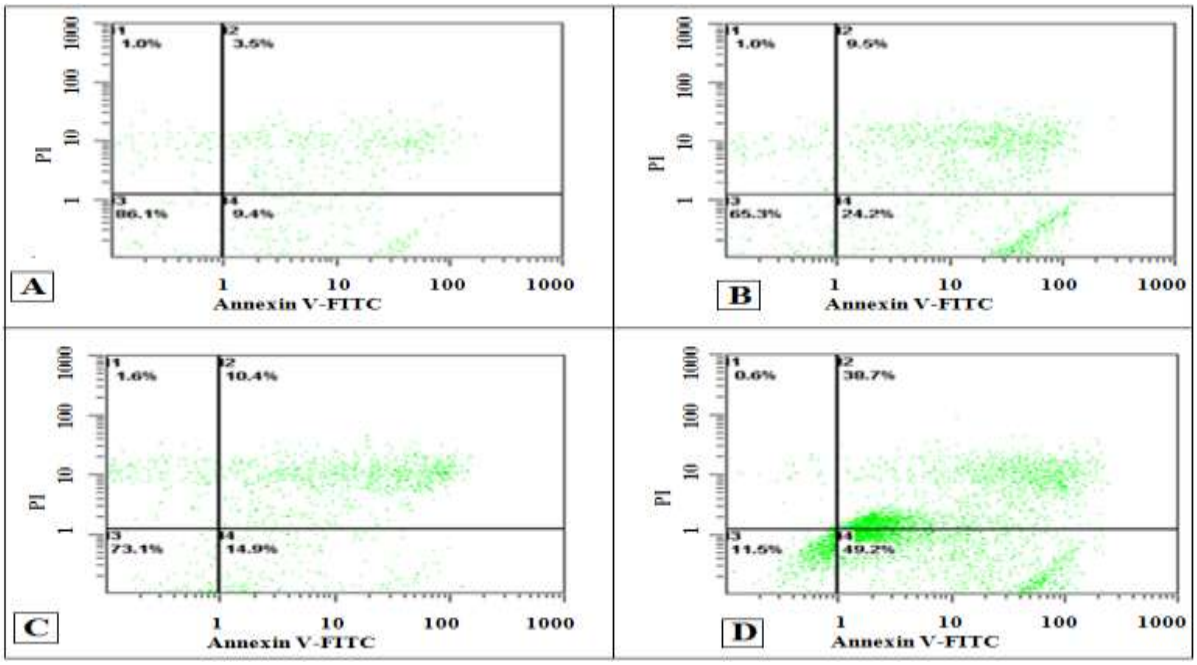

Fig. 3. Annexin-V and PI double-staining FCM of SW480 cells following 24 h incubation with (A) control, (B) 50 μg/ml (5-FU), (C) 50 μg/ml (PB3A), (D) 100 μg/ml (PB3A).

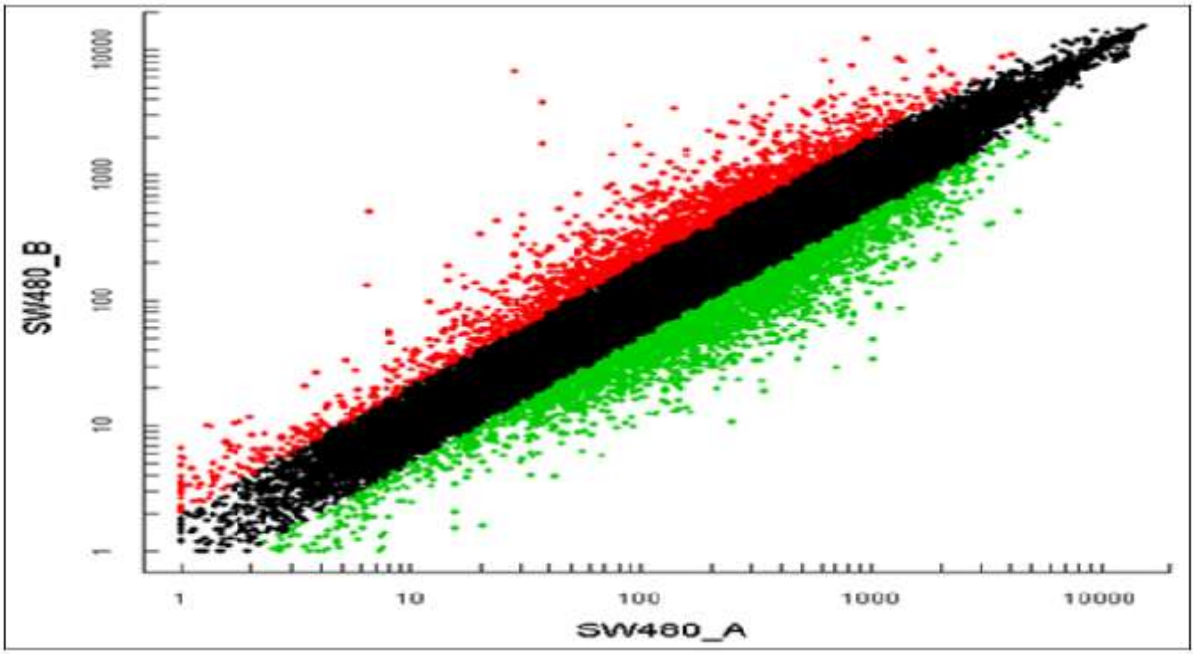

Fig. 4. PB3A induces differential expression of gene signal scatter gram in SW480 cell. Wherein the each of the two sample fluorescence signal intensity values is reported in X-axis and Y-axis coordinate, SW480_A for the control group, SW480_B for the PB3A treated group, the data of red mark and green mark in Fig represents the value of the ratio SW480-B/SW480-A ≥ 2 and ≤ 0.5 respectively, is a differentially expressed gene, the black mark indicates that the value of the ratio SW480-B/SW480-A is between 0.5 and 2 show two groups of cells the level of gene transcription is basically no difference.

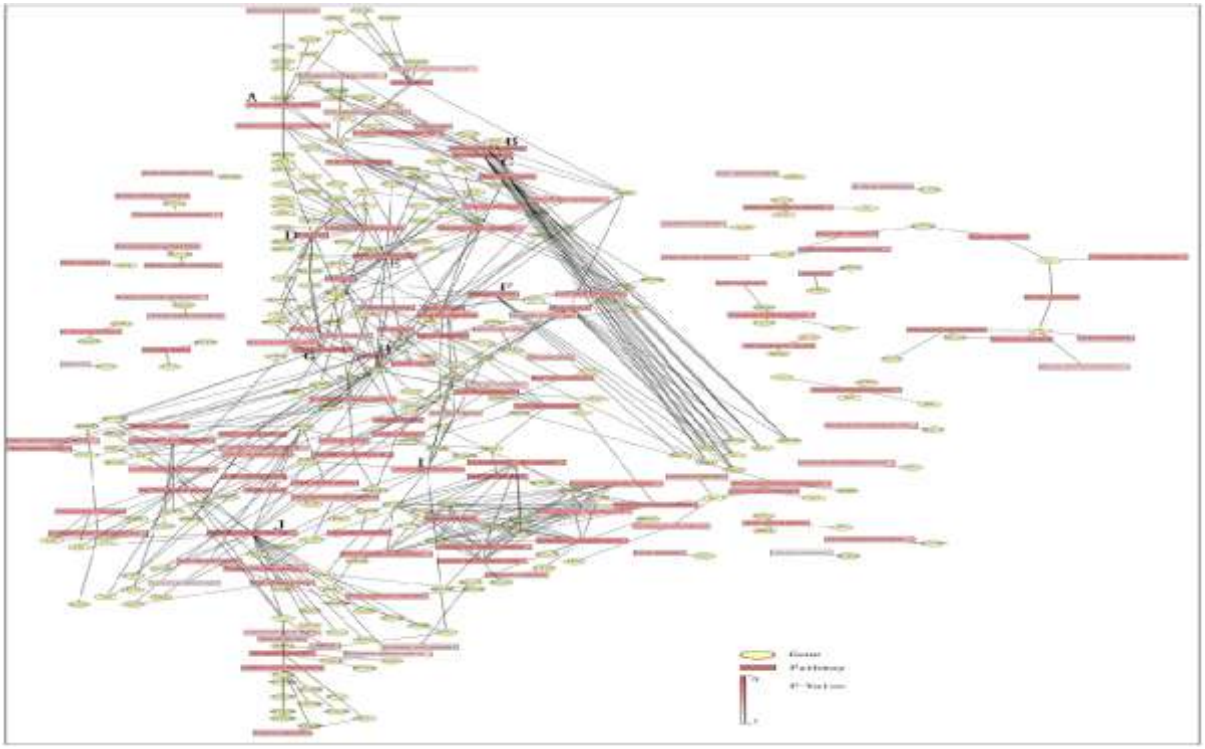

Fig. 5. PB3A induces SW480 cell differential expression genes interaction pathway network. Diagram A, B, C, D, E, F, G, H, I and J shows that TGF-β, Hedgehog pathway, Wnt pathway, cell cycle regulation, MAPK signaling pathway, colorectal carcinoma, p53, apoptosis, calcium and cytokine receptor interactions and other biological processes or signal transduction pathways respectively.

In conclusion, our results clearly demonstrated that PB3A causes tumor cell disruption and subsequent induction of apoptosis, furthermore, the PB3A dosage showed a little enhancement effect on 5-FU cytotoxicity when analyzed in this *in vitro* system. The Gene Chip analysis displayed that PB3A anticancer effects may closely correlate with a wide array of pivotal cell signal transduction or gene expression regulation pathways, which may co-exist with a variety of targets, pathways and effects of the anticancer mechanism. PB3A displays strong anticancer activity against colon cancer cells *in vitro*, and an enhanced effect was observed when PB3A and 5-FU were used in combination which caused a specific inhibitory effect against colon cancer cell SW480, which could be attributed, in part, to its inhibition of proliferation and induction of apoptosis in cancer cells through up-regulation and down-regulation of multiple genes. Therefore, PB3A is a promising anticancer agent with potential for further drug development, particularly in combination with 5-FU.

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