

# Gene Expression Profiling to Delineate the Anticancer Potential of a New Alkaloid Isopicrinine From *Rhazya stricta*

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## Abstract

**Background:** *Rhazya stricta* has been used as a folkloric medicinal herb for treating various diseases such as diabetes, inflammatory disorders, and sore throat. Several studies have revealed the potential of this plant as an important source of phytochemicals with anticancer properties. **Objective:** The present study was designed to isolate a novel anticancer compound from *Rhazya stricta* and elucidate its mechanism of action using genomics approach. **Methods:** *Rhazya stricta* leaves extract was prepared, and several alkaloids were purified and characterized. These alkaloids were screened for their anticancer potential. One of the alkaloids, termed as isopicrinine, showed efficient cytotoxicity against MCF7 breast cancer cell line and was selected for further analysis. RNA-Seq transcription profiling was conducted to identify the affected genes and cellular pathways in MCF7 cells after treatment with isopicrinine alkaloid. **Results:** In vitro studies revealed that newly identified isopicrinine alkaloid possess efficient anticancer activity. Exposure of MCF7 cells with isopicrinine affected the expression of various genes involved in p53 signaling pathway. One of the crucial proapoptotic genes, significantly upregulated in MCF7 after exposure to alkaloid, was *PUMA* (p53 upregulated modulator of apoptosis), which is involved in p53-dependent and -independent apoptosis. Moreover, exposure of sublethal dose of isopicrinine alkaloid in breast cancer cell line led to the downregulation of survivin, which is involved in negative regulation of apoptosis. Besides, several genes involved in mitosis and cell proliferation were significantly downregulated. **Conclusion:** In this article, we report the determination of a new alkaloid isopicrinine from the aerial parts of *Rhazya stricta* with anticancer property. This compound has the potential to be developed as a drug for curing cancer.

## Keywords

*Rhazya stricta*, alkaloids, isopicrinine, anticancer, RNA-Seq, gene expression profile

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## Introduction

*Rhazya stricta* has been used traditionally for the treatment of various diseases in many Middle East and South Asian countries. *R. stricta* is known to be a rich source of several potent compounds with novel structures and with wide variety of pharmaceutical applications for treatment of various diseases such as diabetes, inflammatory diseases, sore throat, helminthiasis, arthritis, and cancer.<sup>1</sup> *R. stricta* produces a large number of indoloterpenoid alkaloids. More than 100 alkaloids with diverse structure and pharmacological properties have been purified and characterized from the leaves, root, stem, and fruits of *R. stricta*.<sup>2-4</sup>

Plant alkaloids are a diverse group of chemical compounds that contain a ring structure and nitrogen atoms.

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More than 27 683 alkaloids have been identified from various natural resources as mentioned in the Dictionary of Natural products. However, only 53 alkaloids have been developed successfully as drugs for therapeutic applications ranging from cough suppressants to antimalarial agents. Vast majority of alkaloids from natural resources are yet to be explored and developed as potent drugs for treatment of various diseases in human. Alkaloids are known to have wide variety of pharmacological activities and have been used as therapeutics for treatment of various diseases such as malaria (eg, quinine), asthma (ephedrine, theobromine, theophylline), hypertension (rescinnamine, reserpine), and cancer (vinblastine, vincristine, homoharringtonine).

*Rhazya stricta* is a rich source of various alkaloids with diverse structure and function. Some of the alkaloids isolated from *R stricta*, such as akuammidine, rhazimanine, stemmadenine, strictanol, and tetrahydrosecaminediol, have shown potential antimicrobial activity against various bacterial pathogens. These alkaloids, which are predominantly present in the leaves of the plants, have shown potent antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Several studies have demonstrated the anticancer, antioxidant, and free radical scavenging properties of the phytochemicals present in *R stricta*.<sup>2,4</sup> Leaf extract of *R stricta* has been shown to induce apoptosis in various cancer cell lines including breast, brain, and lung cancer cell lines.<sup>5,6</sup> Alkaloids such as sewarine, vallesiachotamine, tetrahydrosecamine, tetrahydrosecaminediol, and strictanol have been shown to possess anticarcinogenic properties.<sup>3,7</sup> Rhazinilam has been shown to exert cytotoxic activity by inhibiting microtubule assembly and promoting the growth of abnormal tubulin spirals.<sup>8</sup>

The main objective of our research work is to evaluate the therapeutic potential of new alkaloid isopicrinine from *R stricta* for treatment of cancer and delineate its mechanism of action by analyzing the differential gene expression changes due to the drug action.

## Materials and Methods

### Extraction and Purification of Alkaloids From *Rhazya stricta*

The plant leaves were collected from the Bahrah region, 30 km southeast Jeddah, Saudi Arabia. The identity of the plant was authenticated by a senior botanist, and a sample was deposited at our herbarium repositories in the Department of Biological Sciences, King Abdulaziz University, Jeddah, Saudi Arabia.

### Preparation of Alcoholic Extract of *R stricta* Leaves and Purification of Indole Alkaloids

*Rhazya stricta* leaves were dried in a dark room. After that, the sample (1 kg) was crushed and extracted by chloroform:

ethanol (1:2, v/v) at room temperature for 3 times. The extract was concentrated under a reduced pressure to obtain a dark brown residue (102 g). The total extract was then acidified with a solution of 2% HCl and then extracted again by chloroform to remove the nonbasic compounds. The aqueous layer was basified by ammonium hydroxide solution and was then extracted by chloroform. The chloroform extract was concentrated and dried over sodium sulfate anhydrous to obtain 20.88 g of crude alkaloids.

Aluminum oxide column (800 g, 80 × 2.5 cm) was used to purify individual alkaloids. Crude alkaloids mixture (20 g) was homogenized with aluminum oxide (80 g) and poured onto the top of the column and was equilibrated with chloroform. The eluents were used successively (chloroform, chloroform:ethyl acetate, chloroform:methanol). Fractions were collected (50 mL), and thin-layer chromatography (TLC) was performed using silica gel plates, and Dragendorff's reagent was used as spraying agent. If the material was not homogeneous, preparative TLC (PTLC) was applied using the appropriate solvent system.

### Preparation of Aqueous Extract of *Rhazya stricta* and Purification of Indole Alkaloids

The plant leaves were soaked in 2% HCl solution for 3 days followed by filtration and extraction by chloroform. The aqueous layer was basified by NH<sub>4</sub>OH and then extracted by chloroform. The organic layer was evaporated under reduced pressure to obtain 15 g of crude alkaloids.

Aluminum oxide column (750 g, 75 × 2.5 cm) was used to purify indole alkaloids. The crude alkaloid extract (15 g) was homogenized with a suitable amount of aluminum oxide and poured onto the top of the column. The eluents were used sequentially, that is, chloroform, chloroform:ethyl acetate, chloroform:methanol. Fractions were collected (50 mL), and TLC was performed using silica gel plates, and Dragendorff's reagent was used as a spraying agent. If the material was not homogeneous, PTLC was applied using the appropriate solvent system.

### Characterization of Isopicrinine (I)

The fractions eluted with a mixture of chloroform: ethyl acetate (8.5:1.5, v/v) were collected and examined by TLC on silica gel plates and visualized by Dragendorff's reagent (orange spot developed). PTLC was applied using chloroform: methanol (9.5:0.5, v/v) to give as a brown oily substance (I) (2 mg,  $R_f = 0.47$ ). Ultraviolet (UV)  $\lambda_{\max}$  (MeOH) 234 and 286 nm; infrared (IR)  $\nu_{\max}$  3300 (NH, st), 3015(=CH, st), 2925(CH, st), 1734 (C=O, st) and 1610, 1464 (C=C, st)  $\text{cm}^{-1}$ ; ESI-MS (electrospray ionization–mass spectrometry) (350 eV),  $m/z$  (relative intensity): 339.1 (100) [M+<sup>1</sup>, C<sub>20</sub>H<sub>22</sub>N<sub>2</sub>O<sub>3</sub>-1], 321.0 (100) [M<sup>+</sup>-C<sub>20</sub>H<sub>21</sub>N<sub>2</sub>O<sub>2</sub>], 309.0 (45) [M<sup>+</sup>-C<sub>19</sub>H<sub>21</sub>N<sub>2</sub>O<sub>2</sub>], 289.0 (75), 279.1 (100), 216.1(25), 202.1(25), 184.1 (10), 144.0 (20), 102.1 (20),

**Table 1.** <sup>1</sup>H-NMR (400 MHz) and <sup>13</sup>C-NMR (100 MHz) Spectral Data of Isopicrinine.

<sup>13</sup> C-NMR			<sup>1</sup> H-NMR		
No.	δ <sub>C</sub>	Multiplet	δ <sub>H</sub>	Multiplet (J Hz)	HMBC
2	108.2	s	—	—	5, 3, 6a, 14b, 21b
3	53	d	3.47	d (4.0)	5, 6a
5	88.1	d	4.76	d (2.8)	21b, 6a, 6b
6a	41.1	t	3.45	d (14.0)	OCH <sub>3</sub> , 16, 5
6b			2.12	m	
7	54.3	s	—	—	6a, 6b, 15, 21a, OCH <sub>3</sub>
8	134.2	s	—	—	12, 6a, 6b
9	125.4	d	7.23	dd (8.8, 1.2)	11
10	120.9	d	6.7	ddd (8.8, 8.8, 1.2)	12
11	129	d	7.07	ddd (8.8, 8.8, 1.2)	9
12	111.8	d	6.74	dd (8.8, 1.2)	10
13	149.8	s	—	—	9, 11
14a	22.4	t	2.04	m	
14b			1.98	m	
15	33	d	3.57	br.s	3, 19, 21a, 21b
16	53.2	d	2.34	d (3.6)	15, 3, 21b
17	174	s	—	—	16, OCH <sub>3</sub>
18	13.2	q	1.47	dd (7.2, 2.4)	
19	122	d	5.46	q (7.2)	18, 21b
20	136.7	s	—	—	3, 16, 18, 21b
21a	47.0	t	3.75	br.d (18.0)	
21b			3.21	d (18.0)	
CO <sub>2</sub> Me	52.1	s	3.64	s	3, 15

Abbreviations: HMBC, heteronuclear multiple bond correlation; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br.s, broad singlet; br.d, broad doublet; dd, double doublets.

and 106.2 (45); <sup>1</sup>H-NMR (Bruker WM 400 MHz) and <sup>13</sup>C-NMR (100 MHz) in CDCl<sub>3</sub>:CD<sub>3</sub>OD (as shown in Table 1).

### Cell Culture

MCF7, a breast cancer cell line, was procured from the American Type Culture Collection and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 µg/mL streptomycin 100 U/mL penicillin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. Human pancreatic cancer cell lines, AsPC1 and PANC1, and normal human WISH cell line were obtained from the American Type Culture Collection. AsPC1 cell line was grown in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 µg/mL streptomycin and 100 U/mL penicillin. PANC1 cell line and normal human WISH cell line were cultured in DMEM supplemented with 10% (v/v) FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin.

### Cell Viability and Apoptosis Assay

MCF7 cells (2 × 10<sup>4</sup> cells/well) were subcultured in 24-well plate and incubated at 37°C in a humidified

incubator with 5% CO<sub>2</sub> for 20 hours. Cells were treated with 10 µg/mL and 100 µg/mL of around 34 purified alkaloid fractions (ie, 14 alkaloid fractions from alcoholic extract and 20 alkaloid fractions from aqueous extract) of *R. stricta* and further incubated for 18 hours. After the drug treatment, cells were analyzed under phase-contrast microscope. Based on the preliminary cytotoxicity data, purified alkaloid isopicrinine was selected for further analysis. MCF7 cells (2 × 10<sup>4</sup> cells/well) were treated with varying doses of isopicrinine, that is, 3 µM, 30 µM, 300 µM, 600 µM, and 1500 µM, at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 20 hours. After the drug treatment, cells were analyzed under phase-contrast microscope. Cytotoxicity was detected at 300 µM concentration onward in all the samples. MCF7 cells (2 × 10<sup>4</sup> cells/well) were treated with varying doses of isopicrinine alkaloid in triplicates, that is, 3 µM, 30 µ, 60 µM, 120 µM, 180 µM, 240 µM, 300 µM, and 600 µM, at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 20 hours, and half maximal inhibitory concentration (IC<sub>50</sub>) was determined. The nuclear morphological changes, which are mainly associated with apoptosis, were analyzed by Hoechst 33342 staining. Briefly, MCF7 cells (2 × 10<sup>4</sup> cells/well) were grown on coverslips and treated with 150 µM of purified

alkaloid for 24 hours. The cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 100% methanol for 15 minutes at room temperature. Cells were washed with DPBS and stained with Hoechst 33342 for 15 minutes at room temperature. The cells were washed thrice with DPBS and analyzed under fluorescent microscope (Nikon, Japan).

Human pancreatic cancer cell lines, PANC1, AsPC1 cells, and normal human WISH cell line ( $1.3 \times 10^4$  cells/well) were subcultured in 96-well plates and incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 24 hours. Varying concentrations of isopicrinine, that is, 0, 30 μM, 75 μM, 150 μM, and 300 μM were, added to the cultured cells and further incubated at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 24 hours. The cell viability was determined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay by following the manufacturer's instructions. Briefly, 20 μL/well of MTT reagent was added to the cells, and plates were incubated for 4 hours. After the incubation, media along with the MTT reagent was removed and 150 μL/well of isopropanol was added and further incubated for 15 minutes on shaker incubator. Spectrophotometer readings were taken at 450 nm and 630 nm. This assay is based on the conversion of yellow tetrazolium salt MTT to purple formazan crystal by metabolically active cells. The amount of formazan produced is proportional to the number of viable cells.

### RNA-Seq Analysis

MCF7 cells ( $1.6 \times 10^5$  cells) were treated with varying doses (sublethal) of purified isopicrinine alkaloid, that is, 30 μM and 150 μM, in triplicates, for 18 hours at 37°C. Total RNA was extracted using TRIzol reagent. Gene expression analyses of drug-treated MCF7 cells, in the presence of sublethal concentrations of the isopicrinine alkaloid, were performed using Illumina HiSeq. Each RNA was ribo-depleted (Ribo-Zero, Epicentre) prior to cDNA library preparation (ScriptSeq epicentre). Each cDNA was separately barcode tagged during library preparation and sequenced in multiplex generating approximately >40 million 100 bp reads per sample for the mammalian cell line. The Illumina reads were mapped to their respective assembled reference transcriptome sequence using the mapping software TopHat and Cufflinks.<sup>9</sup> Illumina read abundances mapping to each reference contig was normalized using the RPKM calculation (reads per Kb per million reads). Differentially expressed transcripts were identified and functionally annotated using a variety of statistical analyses available via Bioconductor in R.<sup>10</sup> Clusters of differentially expressed and cycling transcripts were annotated to identify overrepresented GO/KEGG pathways.<sup>11</sup>

## Results

### Purification and Characterization of Isopicrinine (I)

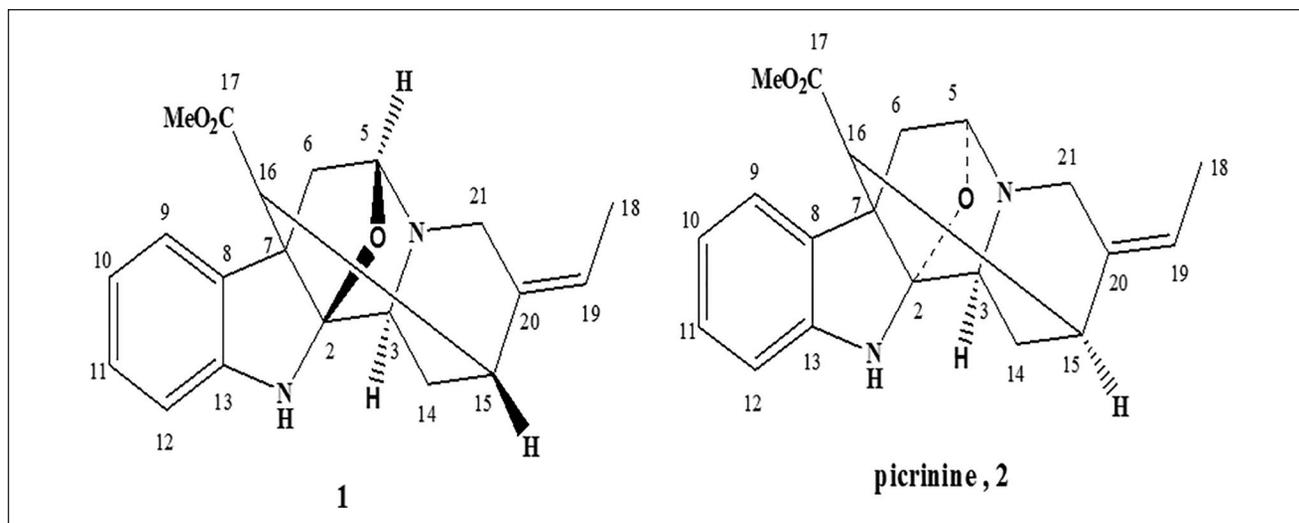
Recent studies have shown the potential of phytochemicals present in the leaves of *R stricta* plant for treatment of cancer. In order to isolate new compounds with anticancer property, total alkaloids extract (alcoholic and aqueous extract) was subjected to column chromatography using aluminum oxide column. A total of 34 individual alkaloids were thus purified and characterized. The purified alkaloids were screened for their cytotoxicity against MCF-7, a breast cancer cell line.

Compound isopicrinine was isolated as an oily material. ESI-MS spectrum of isopicrinine showed a base peak at  $m/z$  339.1, analyzed for the molecular formula, C<sub>20</sub>H<sub>22</sub>N<sub>2</sub>O<sub>3</sub>. The UV spectrum showed absorption maxima at 234 and 286 nm, indicated the presence of indoline ring. The IR spectrum displayed bands at 3300 (NH, st), 3015(=CH, st), 2925(CH, st), 1734 (C=O, st), and 1610, 1463 (C=C, st) cm<sup>-1</sup>. <sup>1</sup>H NMR spectrum exhibited signals for: 4 aromatic protons characteristic for *O*-disubstituted benzene ring resonating at δ<sub>H</sub> 7.23 (dd,  $J = 8.8, 1.2$  Hz, H-9), 6.70 (ddd,  $J = 8.8, 8.8, 1.2$  Hz, H-10), 7.07 (ddd,  $J = 8.8, 8.8, 1.2$  Hz, H-11), 6.74 (dd,  $J = 8.8, 1.2$  Hz, H-12), an olefinic methyl group appeared at δ<sub>H</sub> 1.47 (dd,  $J = 7.2, 2.4$  Hz, Me-18), which was coupled to an olefinic proton signal resonating at δ<sub>H</sub> 5.46 (q,  $J = 7.2$  Hz, H-19) indicating an ethylidene side chain, and a singlet signal at δ<sub>H</sub> 3.64 characteristic for a methyl ester group (Table 1).

The <sup>13</sup>C NMR spectra of isopicrinine, showed resonances of 20 carbons, differentiated by DEPT NMR experiment into 2 methyl (δ<sub>C</sub> 52.1 and 13.2), 3 methylene (δ<sub>C</sub> 47.0, 41.1, and 22.4), 9 methine (δ<sub>C</sub> 129.0, 125.4, 122.9, 122.0, 111.8, 88.1, 53.2, 53.0, and 32.2), and 6 quaternary (δ<sub>C</sub> 174.0, 149.8, 134.2, 108.2, 54.3, and 52.1) carbons.

Resonances attributable to 9 carbons in the <sup>13</sup>C NMR spectrum accounted for 4 carbon-carbon double bonds and 1 carbonyl group, implying that isopicrinine has a hexacyclic skeleton. The association of all unions' carbons and protons were achieved from the heteronuclear single quantum coherence (HSQC) spectrum.

The <sup>1</sup>H-<sup>1</sup>H COSY (correlated spectroscopy) spectrum supported the existence of 1, 2-disubstituted benzene ring through the <sup>1</sup>H-<sup>1</sup>H spin system from H-9 to H-12, an ethylidene group through the correlation between the methine proton resonating at δ<sub>H</sub> 5.46 and the methyl protons at δ<sub>H</sub> 1.47. Furthermore, the <sup>1</sup>H-<sup>1</sup>H COSY spectrum established 3 aliphatic proton sequence H-5-H<sub>2</sub>-6, H-3-H<sub>2</sub>-14-H-15-H-16, and an isolated methylene protons H<sub>2</sub>-21. From COSY and heteronuclear multiple bond correlation (HMBC) spectrum, the correlations of the proton H-3(δ<sub>H</sub> 3.47) with C-2 (δ<sub>C</sub> 108.2) and C-14 (δ<sub>C</sub> 22.4) suggested the linkage



**Figure 1.** Chemical structures of isopicrinine **1** and picrinine **2** alkaloids.

C-2-CH-3-CH<sub>2</sub>-14-CH-15-CH-16. The correlations between H-16 with C-15; C-7 and H-15 with C-20; and C-21 and H<sub>3</sub>-18 with C-20 establish the position of the ethylidene group and the attachments of C-15. The HMBC correlations of H-16 and Me of ester  $\delta_{\text{H}}$  3.64 to C-17 ( $\delta_{\text{C}}$  174.0) indicated the direct connection of H-16 and methyl ester to C-17, the correlation from H-5 to  $\delta_{\text{C}}$  108.2 supported the existence of a C<sub>2</sub>-O-C<sub>5</sub> bond, which was confirmed by its 11 degrees of unsaturation consistent with the molecular formula of C<sub>20</sub>H<sub>22</sub>N<sub>2</sub>O<sub>3</sub>, as established by ESI-MS. The downfield NMR data of CH-5 allowed the attachment of an oxygen atom and nitrogen atom (N-4) to C-5. The HMBC correlation between  $\delta_{\text{H}}$  3.21 H-21 and C-5 revealed the connection of N-4 with C-21, correlation between H-21 and H-18 to C-20  $\delta_{\text{C}}$  136.7. In NOSEY spectrum of compound isopicrinine (**1**), H-15 ( $\delta_{\text{H}}$  3.57) is correlated with H-16 ( $\delta_{\text{H}}$  2.34) and H-3 ( $\delta_{\text{H}}$  3.47) is correlated with H-5 ( $\delta_{\text{H}}$  4.76). Comparison between the above results and literature data, isopicrinine (**1**) is greatly similar to picrinine (**2**).<sup>12</sup> The main differences are in the stereochemistry of C-5 and C-15, where H-3 and H-5 occupy  $\alpha$  position while H-15 with H-16 have  $\beta$  position. So, (**1**) is a new compound and we have named it isopicrinine (Figure 1). The approximate yield of purified isopicrinine alkaloid was 2 mg after processing 1 kg of dry leaves of *R. stricta*.

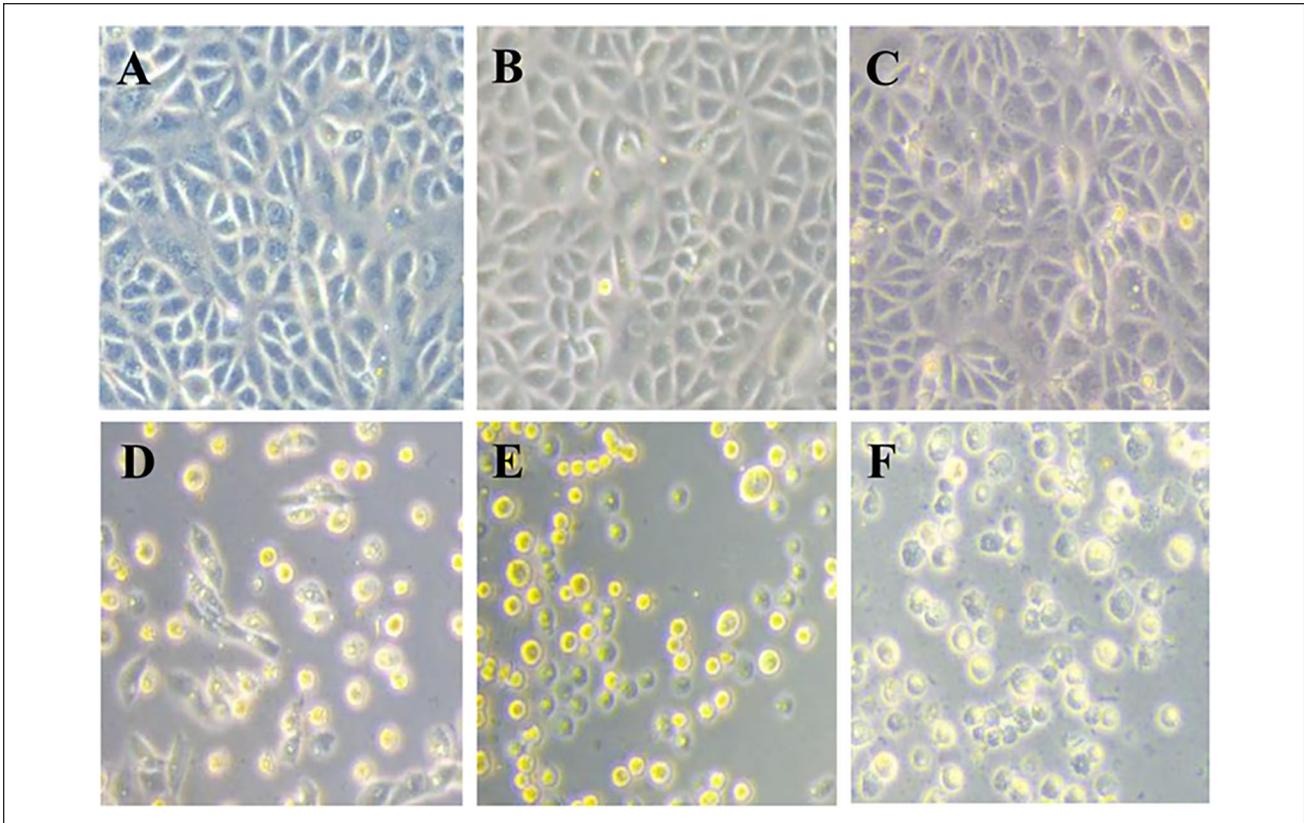
### Anticancer Activity of Alkaloid Isopicrinine (**1**)

Compound **1**, that is, isopicrinine, showed efficient cytotoxic activity with IC<sub>50</sub> value of 240  $\mu\text{M}$ . As depicted in Figure 2D-F, cell shrinkage, cellular detachment, and loss of adherent morphology was seen after treatment with high doses of isopicrinine alkaloid as compared with dimethyl sulfoxide (DMSO)-treated cells (Figure 2A), indicating its anticancer potential. No cytotoxicity was detected at low

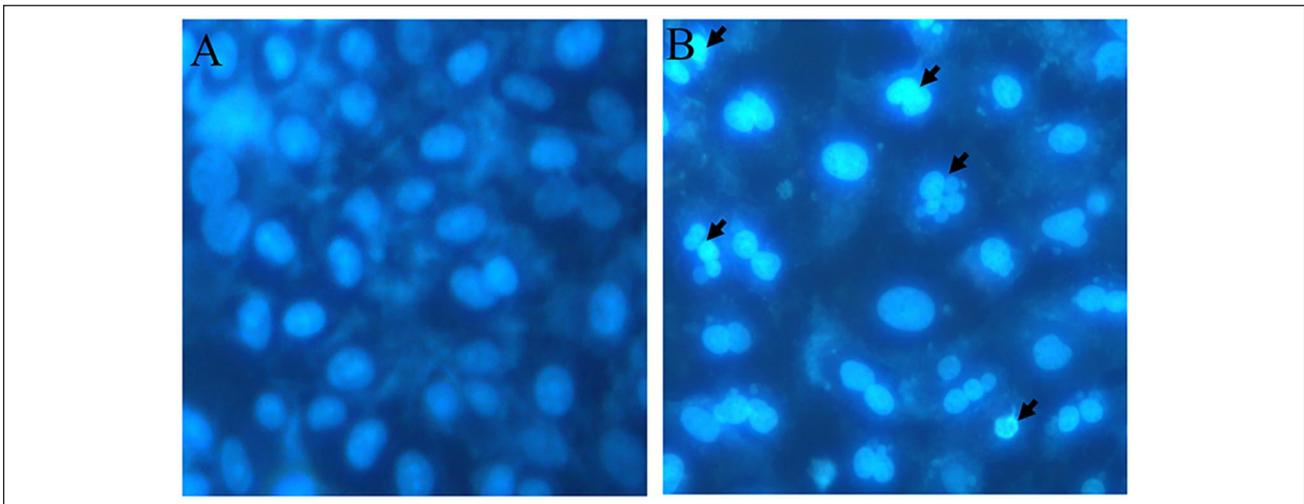
doses of the alkaloid (Figure 2B and C). Hoechst 33342 staining revealed the chromatin condensation and nuclear fragmentation in MCF7 cells after treatment with isopicrinine, which are important features of apoptotic cell death (Figure 3). Isopicrinine also showed efficient cytotoxic activity against pancreatic cancer cell lines AsPC1 and PANC1 as shown in Table 2. However, no significant cytotoxicity was observed in normal human WISH cells after treatment with isopicrinine (Table 2).

### Differential Gene Expression Profiling Using RNA-Seq

MCF7 cell line was treated with sublethal doses of 30  $\mu\text{M}$  and 150  $\mu\text{M}$  of isopicrinine alkaloid. No cytotoxicity was detected at these tested doses. RNA-Seq was performed for analyzing the transcriptome profile in alkaloid-treated MCF7 cells in order to evaluate the pharmacological effects of the new alkaloid isolated from *R. stricta*. To identify the differentially expressed genes after treatment with isopicrinine alkaloid, RNA was extracted from alkaloid-treated MCF7 cells (30  $\mu\text{M}$  and 150  $\mu\text{M}$  doses) and DMSO-treated control cells, and RNA-Seq experiment was carried out. The sequencing data were compared and analyzed to identify the differentially expressed genes in mammalian cells after treatment with isopicrinine alkaloid. Compared with the DMSO-treated control cells, 148 genes were upregulated (fold change > 1;  $P < .05$ ) and 64 genes were downregulated (fold change < -1;  $P < .05$ ) in MCF7 cells treated with 30  $\mu\text{M}$  of isopicrinine. Moreover, treatment of MCF7 cells with 150  $\mu\text{M}$  of isopicrinine resulted in the upregulation of 36 genes (fold change > 1;  $P < .05$ ) and downregulation of 59 genes (fold change < -1;  $P < .05$ ), as compared with the DMSO-treated control cells (Tables 3 and 4; Figure 4).



**Figure 2.** MCF7 breast cancer cell line treated with varying doses of isopicrinine alkaloid. (A) Dimethyl sulfoxide (DMSO) control, (B) 30  $\mu\text{M}$ , (C) 150  $\mu\text{M}$ , (D) 300  $\mu\text{M}$ , (E) 600  $\mu\text{M}$ , and (F) 1500  $\mu\text{M}$ . Cell shrinkage, cellular detachment, and loss of adherent morphology was seen after treatment with high doses of isopicrinine alkaloid.



**Figure 3.** Fluorescent images from Hoechst 33342 staining reveal that isopicrinine alkaloid induced apoptotic cell death. (A) Control MCF7 cells treated with dimethyl sulfoxide (DMSO) alone (B) MCF7 cells treated with 150  $\mu\text{M}$  of isopicrinine. Arrows indicate chromatin condensation and nuclear fragmentation.

Various genes that are involved in DNA replication, cell cycle, and cell proliferation were found to be downregulated in MCF7 cells after treatment with isopicrinine alkaloid

(Table 4 and Figure 4). Genes that are involved in regulation of growth and cell signaling were found to be upregulated. One of the important proapoptotic gene, *PUMA* (p53

**Table 2.** MTT Assay to Determine the Cytotoxic Potential of Isopicrinine Alkaloid on Pancreatic Cancer Cell Lines, PANCI and AsPCI, and Normal Human WISH Cell Line.

No.	Isopicrinine Dose ( $\mu$ M)	PANCI Cell Line % Cell Viability	AsPCI Cell Line % Cell Viability	WISH Cell Line % Cell Viability
1	0	100	100	100
2	30	74.52	87.71	100
3	75	70.75	55.90	100
4	150	69.62	53.49	98.91
5	300	64.6	42.41	81.55

**Table 3.** Upregulated Genes in MCF7 Breast Cancer Cells After Exposure to Isopicrinine Alkaloid Derived From Leaves of *Rhazya stricta*.

Functional Role	Gene	Log2 (Fold Change)	P	Significant
Induction of apoptosis	<i>INHBA</i>	1.02564	.0002	Yes
	<i>MAGED1</i>	0.895445	5.00E-05	Yes
	<i>BBC3</i>	1.48064	5.00E-05	Yes
	<i>CDKN1A</i>	1.11896	5.00E-05	Yes
Response to DNA damage stimulus	<i>GADD45A</i>	1.33298	5.00E-05	Yes
	<i>BTG2</i>	1.48468	5.00E-05	Yes
	<i>DDB2</i>	1.19055	5.00E-05	Yes
	<i>CCND1</i>	1.49047	.00015	Yes
Regulation of transcription	<i>MXI1</i>	1.11765	.00015	Yes
	<i>EGR1</i>	1.39018	5.00E-05	Yes
	<i>ATF3</i>	1.3961	.00025	Yes
	<i>FHL2</i>	1.02376	5.00E-05	Yes
	<i>MDM2</i>	1.72451	5.00E-05	Yes
	<i>NRG1</i>	0.882487	.00015	Yes
Regulation of cell growth	<i>SPP1</i>	1.07532	5.00E-05	Yes
	<i>LTBP4</i>	1.50385	.0001	Yes
	<i>VGF</i>	0.947189	5.00E-05	Yes
Cytoskeleton	<i>AGBL2</i>	2.62717	.0003	Yes
	<i>KRT81</i>	1.79689	.0004	Yes
	<i>DLG2</i>	2.26801	5.00E-05	Yes
	<i>PTP4A1</i>	1.33147	5.00E-05	Yes
	<i>DRP2</i>	2.04721	5.00E-05	Yes

upregulated modulator of apoptosis), was upregulated in MCF7 breast cancer cell line after treatment with the alkaloid (Figure 5). Similarly, *Mxi1* gene that acts as a tumor suppressor was found to be significantly upregulated in MCF7 cells. Downregulation of survivin, which is a member of IAP (inhibitor of apoptosis) family, was detected in MCF7 cells after treatment with sublethal dose of alkaloid. Survivin is known to play a key role in negative regulation of apoptosis. Several genes that are involved in cell cycle and cell proliferation, such as *CDC6*, *CDK1*, *CDCA5*, *DLGAP5*, *NUSAP1*, *KIF23*, *PRC1*, *BIRC5*, *AURKB*, *NDC80*, and *CDC45*, were significantly downregulated after the exposure with the sublethal dose of 150 $\mu$ M of isopicrinine alkaloid (Table 4; Figures 5 and 6). However, these genes were not affected at low concentration of 30  $\mu$ M of the alkaloid.

## Discussion

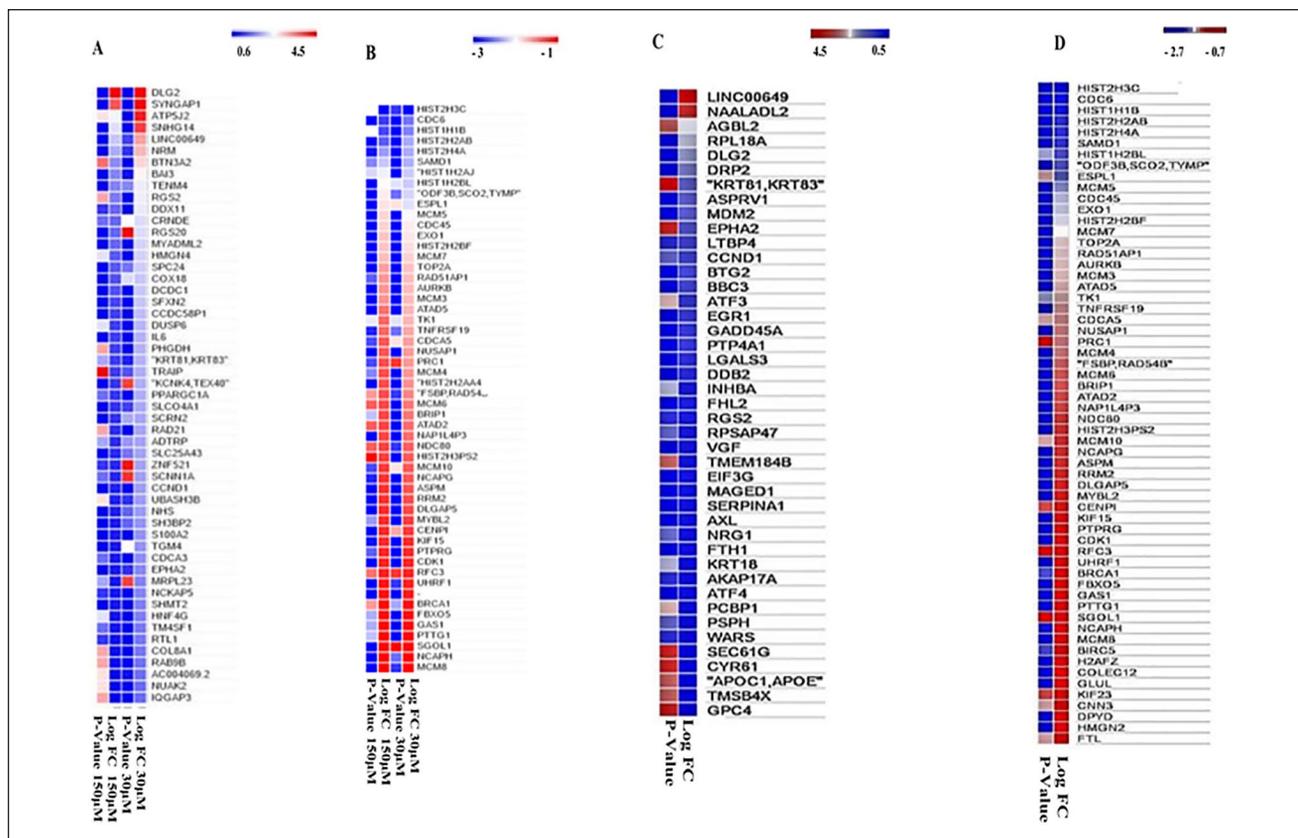
Phytochemical components of *R stricta* is known to contain more than 100 alkaloids with diverse and complex structures with wide range of biological activities. Some of these alkaloids have been shown to possess antibacterial, anticancer, or wound healing activities.<sup>2</sup> We isolated a new alkaloid, termed as isopicrinine, from the leaves of *R stricta*. This purified alkaloid exhibited significant cytotoxic activity for MCF7 breast cancer cell line. RNA-Seq transcription profiling revealed several differentially regulated genes in MCF7 cells after exposure to sublethal dose of the alkaloid. Several genes that play important roles in DNA replication, mitosis, and cell proliferation were significantly downregulated, and some key proapoptotic genes were found to be upregulated, indicating the potential of new alkaloid isopicrinine as an antitumor agent.

**Table 4.** Downregulated Genes in MCF7 Breast Cancer Cells After Exposure to Isopicrinine Alkaloid Derived From Leaves of *Rhazya stricta*.

Functional Role	Gene	Log2 (Fold Change)	P	Significant	
Cell division	<i>CDC6</i>	-2.32211	5.00E-05	Yes	
	<i>CDK1</i>	-1.14933	5.00E-05	Yes	
	<i>CDC45</i>	-1.55071	.0003	Yes	
	<i>ASPM</i>	-1.3045	5.00E-05	Yes	
	<i>NUSAP1</i>	-1.53939	5.00E-05	Yes	
	<i>KIF23</i>	-0.904965	.00035	Yes	
	<i>PRC1</i>	-1.52742	.00045	Yes	
	<i>BIRC5</i>	-0.957158	.0002	Yes	
	<i>AURKB</i>	-1.64598	5.00E-05	Yes	
	<i>NDC80</i>	-1.34275	.0001	Yes	
	<i>SGOL1</i>	-1.04359	.0005	Yes	
	<i>FBXO5</i>	-1.06667	5.00E-05	Yes	
	<i>PTTG1</i>	-1.05005	5.00E-05	Yes	
	<i>NCAPH</i>	-1.01758	.00015	Yes	
	<i>NCAPG</i>	-1.31394	5.00E-05	Yes	
	DNA replication	<i>MCM3</i>	-1.64431	5.00E-05	Yes
<i>MCM4</i>		-1.52185	5.00E-05	Yes	
<i>MCM5</i>		-1.87773	5.00E-05	Yes	
<i>MCM6</i>		-1.42256	5.00E-05	Yes	
<i>MCM7</i>		-1.71581	5.00E-05	Yes	
<i>MCM8</i>		-1.01268	.0001	Yes	
<i>MCM10</i>		-1.32104	.0003	Yes	
<i>TOP2A</i>		-1.66227	5.00E-05	Yes	
<i>CDC45</i>		-1.80081	5.00E-05	Yes	
<i>RFC3</i>		-1.14176	.00045	Yes	
<i>RRM2</i>		-1.2797	5.00E-05	Yes	
Chromosome organization		<i>HIST2H4A</i>	-2.13768	5.00E-05	Yes
		<i>HIST2H2AA4, HIST2H2AC</i>	-1.50511	5.00E-05	Yes
		<i>HIST2H3PS2</i>	-1.3321	5.00E-05	Yes
		<i>HIST2H2BF</i>	-1.75681	5.00E-05	Yes
		<i>HIST2H3C</i>	-2.71641	.0001	Yes
	<i>HIST2H2AB</i>	-2.14409	5.00E-05	Yes	
	<i>HIST1H2BL</i>	-2.03159	.00025	Yes	
	<i>HIST1H1B</i>	-2.27766	5.00E-05	Yes	
	<i>HIST1H2AM, HIST1H3J</i>	-2.03436	5.00E-05	Yes	
	<i>HIST1H2AJ, HIST1H2AK</i>	-2.03436	5.00E-05	Yes	
	<i>DLGAP5</i>	-1.26336	5.00E-05	Yes	
	<i>ESPL1</i>	-1.9647	.0003	Yes	
	<i>H2AFZ</i>	-0.95203	5.00E-05	Yes	
	DNA repair	<i>EXO1</i>	-1.78374	5.00E-05	Yes
		<i>BRCA1</i>	-1.07641	.0002	Yes
		<i>UHRF1</i>	-1.1337	.0001	Yes
<i>RAD51API</i>		-1.65155	5.00E-05	Yes	
Regulation of transcription	<i>ATAD2</i>	-1.38776	5.00E-05	Yes	
	<i>MYBL2</i>	-1.23112	5.00E-05	Yes	
	<i>BRIP1</i>	-1.41617	5.00E-05	Yes	
	<i>ATAD5</i>	-1.62821	5.00E-05	Yes	

One of the important proapoptotic genes, significantly upregulated in MCF7 cells after treatment with isopicrinine alkaloid, was *BBC3* (Bcl-2-binding component 3). This

gene, also known as *PUMA*, is a member of the Bcl-2 protein family and is involved in p53-dependent and -independent apoptosis.<sup>13,14</sup> *BBC3* expression is controlled by tumor



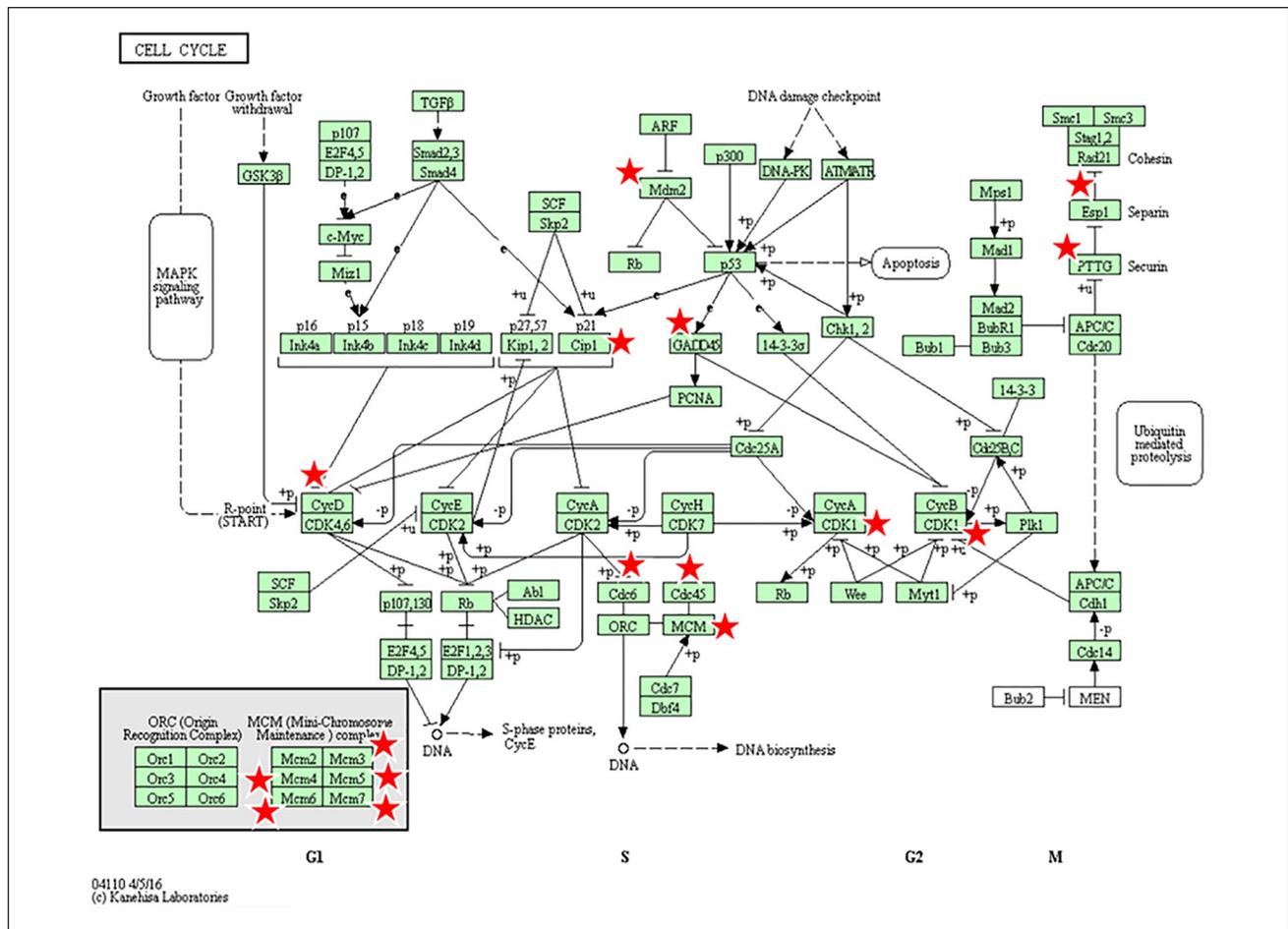
**Figure 4.** Cluster analysis of genes from MCF7 breast cancer cells after treatment with isopicrinine alkaloid compared with dimethyl sulfoxide (DMSO)-treated control cells. (A) Genes upregulated in MCF7 cells after treatment with 30  $\mu$ M and 150  $\mu$ M of alkaloid. (B) Genes downregulated in MCF7 cells after treatment with 30  $\mu$ M and 150  $\mu$ M of alkaloid. (C) Genes upregulated in MCF7 cells after treatment with 150  $\mu$ M of alkaloid. (D) Genes downregulated in MCF7 cells after treatment with 150  $\mu$ M of alkaloid.

suppressor p53, and on activation, it interacts with anti-apoptotic Bcl-2 family members and hence releases Bax or Bak, which leads to mitochondrial outer membrane permeabilization. Following mitochondrial dysfunction, caspases are activated, which finally leads to apoptotic cell death.<sup>15,16</sup> Induction of *BBC3* proapoptotic gene after exposure to sub-lethal dose of the alkaloid clearly suggest its potential as a potential drug target for controlling tumor development.

*Mxi1* gene, which encodes for Max-interacting protein 1, was found to be upregulated in MCF7 cells after treatment with the alkaloid. *Mxi1* protein act as a tumor suppressor and negatively regulate c-Myc activity. Expression of the oncogenic *c-Myc* gene is very strongly controlled in normal cell; however, it is found to be deregulated in various types of human cancer. Myc protein interacts and dimerizes with Max transcription factor and regulates the expression of various genes involved in cell division and apoptosis. *Mxi1* protein inhibit the activity of Myc by competing for Max protein, and hence prevent the tumor growth. Defects in *Mxi1* gene are frequently detected in prostate cancer patients.<sup>17</sup>

Exposure of high dose of isopicrinine alkaloid in MCF7 cells led to the downregulation of *BIRC5* gene, which encodes for survivin, also known as baculoviral IAP repeat-containing 5 (*BIRC5*). Survivin is a member of IAP family and play an important role in negative regulation of apoptosis by inhibiting the caspase activation.<sup>18</sup> Tamm and colleagues have demonstrated that survivin inhibits both Bax- and Fas-induced apoptotic pathways.<sup>19</sup> It has been observed that silencing of survivin expression leads to increase in apoptotic cell death and subsequent decrease in tumor growth. Moreover, expression of survivin protein is found to be highly upregulated in almost all types of human cancer and also in fetal tissue, but it is not expressed in normal adult cell, hence making it an attractive drug target for cancer therapy. Tamm and colleague reported the expression of survivin in all the 60 different human cancer cell lines that are being used in the National Cancer Institute's cancer drug screening program, with the maximum level of expression in breast and lung cancer cell lines.<sup>19</sup> The mechanism of survivin regulation are still not well characterized, but its regulation seems to be linked to p53 protein and is





**Figure 6.** KEGG pathway analysis of differentially regulated genes in MCF7 cells after treatment with isopicrinine alkaloid, depicting the affected genes that play significant role in cell cycle and cell proliferation. Modulated genes are marked with red star.

protein that is key regulator of cytokinesis. It is required to recruit PLK1 and KIF14 to the central spindle. It has been shown to act as an oncogene for promoting cell proliferation, inhibition of apoptosis, and tumor progression in bladder cancer.<sup>26</sup>

An interesting gene found to be downregulated by sublethal dose of isopicrinine alkaloid is *NUSAP1*, which is involved in mitotic spindle assembly, chromosome segregation during cell division and cytokinesis. Recent study has revealed the role of *NUSAP1* in BRCA1-regulated pathways of DNA repair and centrosome duplication, which is essential for maintaining genome stability.<sup>27</sup> Several studies have demonstrated the overexpression of *NUSAP1* in various cancer such as melanoma, prostate, glioblastoma, and liver carcinoma.<sup>27,28</sup> It has been linked to enhanced disease aggression in meningiomas, increased risk in breast cancer patients, and development of resistance to chemotherapy in pancreatic cancer patients. It has now been identified as an important biomarker for breast ductal carcinoma and development of drug resistance.

*Ndc80* gene, found to be downregulated by isopicrinine alkaloid, encodes for a kinetochore protein that is responsible for correct chromosome segregation during mitosis by ensuring the proper bipolar attachment of chromosome. Overexpression of *Ndc80* has been shown to induce hepatitis B virus-related hepatocellular carcinoma. RNAi-mediated silencing of *Ndc80* expression in hepatoma cell line HepG2 resulted in inhibition of cell proliferation and suppressed the replication of hepatitis B virus.<sup>29</sup> *CDC45* gene encodes for sororin protein, which is required for sister chromatid cohesion in interphase. Phosphorylation and activation of sororin by ERK had been shown to play a key role in cell proliferation in lung cancer.<sup>30,31</sup> Aurora B kinase is required for the attachment of mitotic spindle to the centromere during cell division. Overexpression of this kinase leads to unequal chromosomal separation, resulting in abnormal number of chromosomes in the daughter cells, thus causing cancer development.<sup>32,33</sup> *PTTG1* (pituitary tumor-transforming 1) gene found downregulated after exposure with isopicrinine alkaloid, encodes for a protein

that inhibit separins from promoting sister chromatid separation. This protein has been shown to have tumorigenic activity in animal models and is also highly upregulated in several tumors.<sup>34,35</sup> Our gene expression profiling data have revealed that novel alkaloid isopicrinine, isolated from medicinal herb *R stricta*, exert its anticancer activity by targeting the p53 pathway, and thus causing the inhibition of various genes involved in cell proliferation. Our earlier studies have also demonstrated that medicinal plant *R stricta* is an important resource for discovering and isolating novel compounds with potential anticancer activity.<sup>4,36</sup>

## Conclusion

In conclusion, our data clearly indicate that exposure of MCF7 breast cancer cells with sublethal dose of isopicrinine alkaloid affected the expression of various genes involved in p53 signaling pathway. One of the crucial proapoptotic genes, significantly upregulated in MCF7 after exposure to alkaloid, was *PUMA*, which is a member of the Bcl-2 protein family, and is involved in p53-dependent and p53-independent apoptosis. Moreover, exposure of sublethal dose of isopicrinine alkaloid in breast cancer cell line led to the downregulation of survivin, which is involved in negative regulation of apoptosis. Besides, inhibition of several genes involved in mitosis and cell proliferation indicate the potential of isopicrinine alkaloid from *R stricta* leaves as a new cancer agent.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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