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## Inhibition of mTORC1 activity by natural ingredient of Ignatius beans

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### ABSTRACT

This study screened a collection of >2,800 naturally occurring products and identified Ignatius beans extract capable of inhibiting mTORC1 activity. HeLa cells were treated with aqueous extract from Ignatius beans to assess the activity of mTORC1. Treatment of HeLa cells with Ignatius bean extract inhibits the enzymatic activity of mTORC1 as assessed by the phosphorylation of p70 S6K (S6K) at Thr 308 in HeLa cells. This plant seed extract also exerts inhibitory effects on the activation phosphorylation of Akt. In addition, flow cytometry analysis revealed that Ignatius bean extract causes HeLa cells to accumulate in G2/M phase of cell cycle. Trypan blue dye exclusion assay was carried out to determine the cytotoxicity of Ignatius Beans. The plant extract was not overtly cytotoxic at doses that inhibit mTORC1 activity. These data suggest that Ignatius bean extract could be used as a potent inhibitor of cell growth and cell proliferation. © 2012 Trade Science Inc. - INDIA

### KEYWORDS

Ignartius beans;  
Cell cycle;  
mTORC1;  
Akt;  
G2/M.

### INTRODUCTION

Ignatius beans, plant seeds of *Strychnos Ignatii*, have long been used as a remedy for treatment of neurological disorders such as anxiety symptoms, nerve depression, and insomnia<sup>[1]</sup>. This study describes the inhibition of mTORC1 by the natural ingredient from Ignatius beans.

mTOR is an evolutionarily conserved Ser/Thr protein kinase that controls many cellular processes such as translation, cell proliferation, cell cycle progression, cell size regulation, transcription, and cytoskeleton regulation in response to a variety of environmental changes<sup>[2]</sup>. Two multiple protein complexes of mTOR have been known. Unique accessory proteins distin-

guish the two complexes. Raptor and Rictor define mTORC1 and mTORC2, respectively<sup>[3]</sup>. mTORC1 is activated by phosphoinositide 3-kinase (PI3K) and its downstream Akt and upregulates anabolic processes such as protein synthesis, cell size, and downregulates catabolic processes such as autophagy<sup>[3,4]</sup>. mTORC1 activity is also controlled by AMP-activated protein kinase (AMPK), which is activated under low cellular energy status<sup>[4,5]</sup>. Much less is known about mTORC2 function. mTORC2 which promotes cell survival and cytoskeleton organization, is a protein kinase that phosphorylates AGC kinase family members including Akt, Serum- and Glucocorticoid-regulated kinase (SGK), and protein kinase C (PKC)<sup>[6,7]</sup>.

Rapamycin is a well-known natural antibiotic that

acts as a specific allosteric inhibitor of mTORC1 protein kinase<sup>[8]</sup>. The ability of rapamycin to suppress mTORC1 has suggested that rapamycin and its analogs could serve as a potential therapeutic agents for human diseases associated with the dysregulation of mTORC1 signaling<sup>[9]</sup>. Although rapamycin effectively inhibits cell proliferation and angiogenesis by inhibiting mTORC1 in some human tumors<sup>[10,11]</sup>, rapamycin has crucial disadvantage as an anti-cancer drug. This has been majorly attributable to the presence of negative feedback loop by which inactivated mTORC1 activates PI3K/Akt pathway, resulting in the activation of cell survival pathway<sup>[12-14]</sup>.

In search of naturally occurring plant products that can inhibit mTORC1 activity, a large collection of >2,800 naturally occurring products was screened. This study has found that an aqueous extract from Ignatius beans inhibits mTORC1 activity as well as PI3K/Akt pathway resulting the accumulation of cell cycle at G2 to M phase in cultured human HeLa cells.

## EXPERIMENTAL

### Cell culture and reagents

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Antibody for tubulin was purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal antibody for S6K was from Santa Cruz (Santa Cruz, CA, USA). Antibody against phosphorylated S6K at Thr 389 was from Cell Signaling (Boston, MA, USA). Rabbit antibodies for phosphorylated Akt at Thr 308 and Ser 473 were also from Cell Signaling.

### Preparation of aqueous extract from Ignatius beans

Beans of *Strychnos Ignatii* were obtained from Natural Medicine Research Center of Korea Institute of Bioscience and Biotechnology, Daejeon, South Korea. The plant seeds were identified by a botanist from the herbarium of Daegu Haany University. Ignatius beans (600g) were pulverized, washed with water three times. The fresh portion was collected and dried at 40 °C in a hot air oven. The plant material was homogenized in 4

L of hot boiling water for 2 h. After filtration through Whatman No. 1 paper, the filtrate was lyophilized. The lyophilized aqueous extract was used to evaluate its effects on mTORC1 activity, cell cycle progression, and cell viability.

### Analysis of mTORC1 signal pathway

Effect of Ignatius bean extract on mTORC1 activity in cultured HeLa cells was determined by monitoring the phosphorylation of S6K, one of prominent mTORC1 substrates, by Western blot analysis using anti-phospho-S6K antibody. Activation of Akt in HeLa cells was assessed evaluating the phosphorylation at Thr 308 by Western blotting using antibody directed against the phosphorylated Akt.

### Cell cycle analysis

HeLa cells were treated with Ignatius bean extract (50-100 µg/ml) for 24 h, fixed with ice-cold 70% ethanol, and incubated on ice for 30 min. Fixed cells were then treated with RNaseI and stained with propidium iodide. DNA contents of cells were measured by flow cytometry<sup>[15]</sup>.

### Western blotting

Levels of S6K, Akt, and their phosphorylated proteins in HeLa cells treated with concentrations of Ignatius bean extract were assayed by Western blotting. Cellular proteins were resolved on a 10% acrylamide gel. Resolved proteins were subjected to electroblotting onto nitrocellulose membrane, blocked with 5% (w/v) skim milk, and incubated with respective antibody.

### Cell viability assay

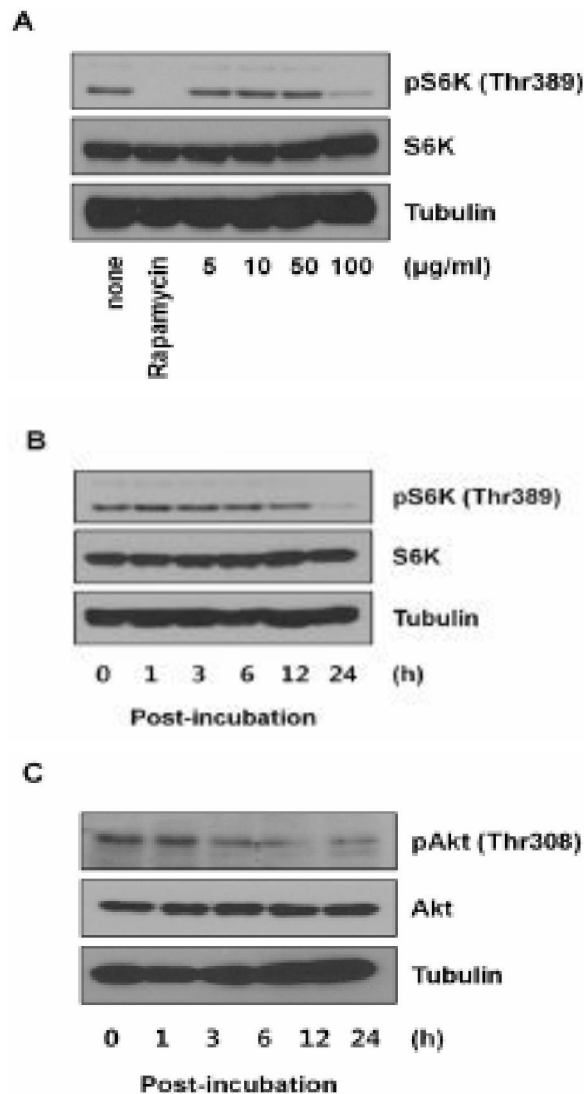
Viability of HeLa cells after treatment with Ignatius bean was determined by Trypan blue dye exclusion method as described<sup>[16]</sup>.

## RESULTS & DISCUSSION

### Inhibition of mTORC1 by an aqueous extract from Ignatius beans

S6 Kinases (S6K1 and S6K2) as well as eIF4E-binding proteins (4E-BPs)<sup>[17]</sup> are predominant substrates of mTORC1. The ability of Ignatius bean extract to inhibit mTORC1 was assessed by examining the phosphorylation of S6K1 in HeLa cells treated with

## FULL PAPER



**Figure 1 : Ignatius bean extract inhibits mTORC1 activity in HeLa cells. (A)** HeLa cells ( $10^6$ ) were treated with Ignatius bean extract (5, 10, 50, or 100 µg/ml) or rapamycin (100 nM) for 24 h. Western blot analysis was performed by using antibody against phosphorylated S6K to examine the phosphorylation of S6K as a measure of mTORC1 activity. **(B)** HeLa cells were treated with 100 µg/ml Ignatius bean extract for the indicated amount of time. Cells were examined for the phosphorylation of S6K at Thr 389. **(C)** HeLa cells were treated with 100 µg/ml Ignatius bean extract for the indicated amount of time. Western blot analysis was performed to examine the phosphorylation of Akt at Thr 308.

various concentrations of Ignatius bean extract. The activity of mTORC1 was inhibited by Ignatius bean extract at a high concentration of 100 µg/ml (Figure 1A). Time dependent analysis of the effect of Ignatius bean extract revealed that mTORC1 activity was inhibited in 24 h after the treatment of cells with 100 µg/

ml Ignatius bean extract (Figure 1B). Akt, which is also called as PKB, is a serine/threonine kinase known to be activated by phosphorylation. Akt is phosphorylated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) and mTORC2 at Thr 308 and Ser 473, respectively. Activated Akt activates mTORC1 by inhibiting TSC1/2, a negative regulator complex of mTORC1<sup>[3]</sup>. Treatment of Ignatius bean extract reduced cellular levels of phosphorylated Akt at Thr 308 in HeLa cells (Figure 1C). This result suggests that the natural ingredient of Ignatius beans may directly inhibit mTORC1 activity or indirectly influence mTORC1 activity through the inhibition of Akt signaling. The inhibition of Akt phosphorylation at Thr308 strongly denies the involvement of negative feedback effect by PI3K/Akt pathway in cells treated with Ignatius bean extract.

### Cell cycle arrest at G2/M phase by Ignatius bean extract

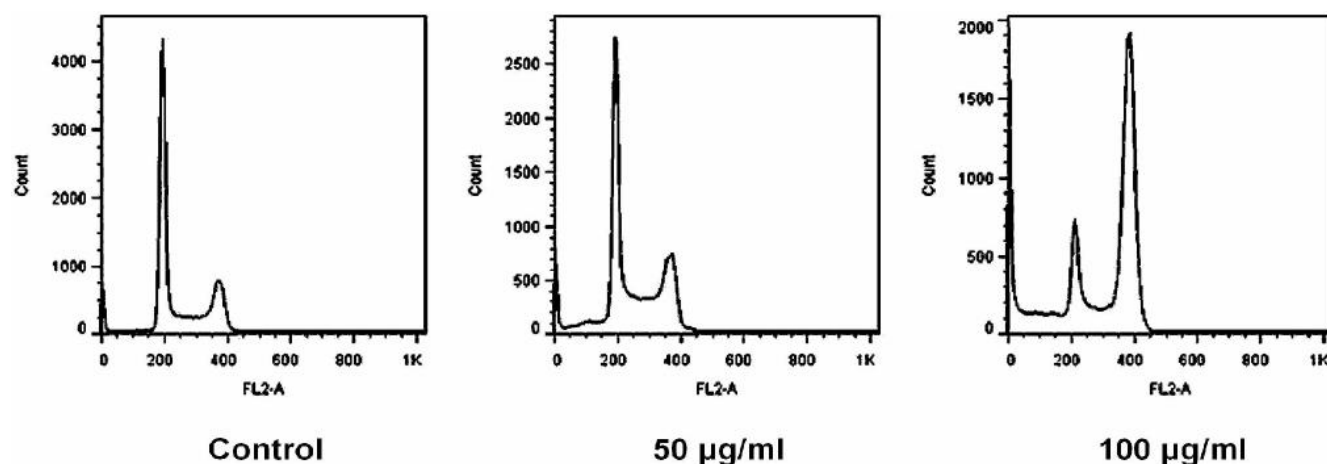
mTOR signaling complex integrates signals from growth factors and nutrient availability to control cell growth and cell division. These cellular events require the accumulation of cell cycle-associated proteins<sup>[15,18]</sup>. There are studies demonstrating a link between mTORC1 signaling and control of mitotic kinases. For example, mTORC1 signaling is required for G1/S progression via translational regulation of cyclins and p21/p27. mTORC1 function is also known to be involved in G2/M progression through the regulation of cellular levels of Cdc2 and Cyclin B<sup>[19]</sup>. Our data analyzing the distribution of cells in different phases of cell cycle by flow cytometry illustrate that the treatment with Ignatius bean extract results in an accumulation of G2/M phase of cell cycle in HeLa cells in a dose- and time-dependent manners (Figure 2).

### Cytotoxicity of Ignatius beans

The cytotoxic activity of the ingredient of Ignatius beans that inhibit mTORC1 activity was evaluated in HeLa cells by Trypan blue dye exclusion method<sup>[16]</sup>. The plant extract was not overtly toxic at doses that can inhibit mTORC1 activity and arrest cell cycle progression (TABLE 1).

### ACKNOWLEDGEMENTS

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Percentages of cell cycle phases

Treatment	G1 (%)	G2-M (%)
Control	60.23	21.38
50 µg/ml for 24 h	43.69	24.25
100 µg/ml for 24 h	15.25	52.10

Figure 2 : Cell cycle analysis of HeLa cells treated with Ignatius bean extract. HeLa cells were treated with 50 or 100 µg/ml Ignatius bean extract for 24 h. Cells were fixed with 70% ethanol and stained with propidium iodide (PI). Cell cycle was analyzed by flow cytometry. Percentages of G1 and G2/M phases of cell cycle were shown.

TABLE 1 : Effect of Ignatius bean extract on viability of HeLa cells. The cytotoxicity of Ignatius bean extract was evaluated in HeLa cells by Trypan blue dye exclusion method<sup>[16]</sup>. HeLa cells were treated with various concentrations of Ignatius bean extract for 24 h. Cells were harvested and stained with 0.4% trypan blue and counted for stained and unstained cells using hemacytometer. Percentages of unstained cells (viable cells) were presented. Values represent means  $\pm$ SD (n  $\geq$  3 experiments).

Treatment (µg/ml)	Viable cells (%)
control	100.0
10	82.5 $\pm$ 9.8
25	85.1 $\pm$ 8.0
50	87.0 $\pm$ 1.2
75	90.1 $\pm$ 5.4
100	83.3 $\pm$ 1.0

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