PRELIMINARY INVESTIGATION OF *MYO*-INOSITOL PHOSPHATES PRODUCED BY ASUIA279PHYTASE ON MCF-7 CANCER CELLS

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ABSTRACT: Phytate or *myo*-inositol hexakisphosphates (IP_6) is widely distributed in plants like rice bran. The production of *myo*-inositol phosphate intermediates has received much attention due to the remarkable potential health benefits offered by the compounds. In this study, the cytotoxicity of the partially purified *myo*-inositol phosphate fractions and commercial IP_1 and IP_6 were investigated against MCF-7 breast cancer cell lines. The study showed that the commercial standard IP_1 and IP_6 showed good inhibition towards the MCF-7 cell line. The MCF-7 cells growth was inhibited in minimum concentration of *myo*-inositol phosphates (<1000 µg/ml). However, no inhibition observed on the MCF-7 cell line by the *myo*-inositol phosphates fractions partially purified from rice bran at concentration <1000 µg/ml. The inhibition of MCF-7 was only observed at concentration more than 30 mg/ml with more than 40% cells were inhibited. This indicates that the partially purified rice bran *myo*-inositol phosphates degraded by ASUIA279 phytase on MCF-7 breast cancer cells exhibit positive results towards the inhibition of cancer cells growth at relatively high concentration.

ABSTRAK: Fitat atau *myo-inositol hexakisphosphate* (IP6) dikenali umum teragih di dalam tumbuhan seperti dedak padi. Penghasilan perantaraan fosfat *myo-inositol* mendapat perhatian memandangkan ia berpotensi tinggi dalam kesihatan. Dalam kajian ini, kesitotoksikan sebahagian daripada fosfat *myo-inositol* separa tulen, IP1 komersil dan IP6 komersil dikaji terhadap produk yang berupa sel kekal (*cell lines*) kanser payu dara MCF-7. Tumbesaran sel MCF-7 direncatkan dalam pekatan minima fosfat *myo-inositol* (<1000 µg/ml). Tetapi, tidak ada perencatan dilihat terhadap sel kekal MCF-7 oleh sebahagian fosfat *myo-inositol* separa tulen daripada dedak padi pada kepekatan <1000 mg/ml. Perencatan MCF-7 hanya dilihat pada kepekatan lebih daripada 30 mg/ml dengan lebih daripada 40% sel terencat. Ini menunjukkan bahawa fosfat *myo-inositol* daripada dedak padi separa tulen terdegradasi oleh fitat ASUIA279 terhadap sel kanser MCF-7 dimana ia menunjukkan keputusan positif terhadap perencatan tumbesaran sel kanser pada kepekatan tinggi.

KEYWORDS:*myo-inositol phosphates; phytase; MCF-7cell line; cancer*

1. INTRODUCTION

Phytate or *myo*-inositol hexakisphosphates (IP_6) is widely distributed in plants, particularly in cereals and legumes, such as corn, soybean, wheat bran, rice bran, cotton seeds, rape seeds and soybean with a concentration range between 0.4% to 6.4% (w/w) and also in mammalian cells at concentrations of 10 µM to 1mM [1]. Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is a phosphomonoesterase that acts on phytate in sequential and stepwise manner, releasing inorganic orthophosphate (Pi) and yielding partially phosphorylated *myo*-inositol phosphates which may again become substrates for further hydrolysis (Fig. 1) [2]. It catalyzes the degradation of phytic acid into lower *myo*-inositol phosphates; pentakis- (IP_5), tetrakis- (IP_4), tris- (IP_3), bis- (IP_2), and mono phosphate/s (IP_1); with the release of inorganic orthophosphate (Pi) in sequential manner [3]. Phytases are found naturally in plants and microorganisms, particularly fungi [4].

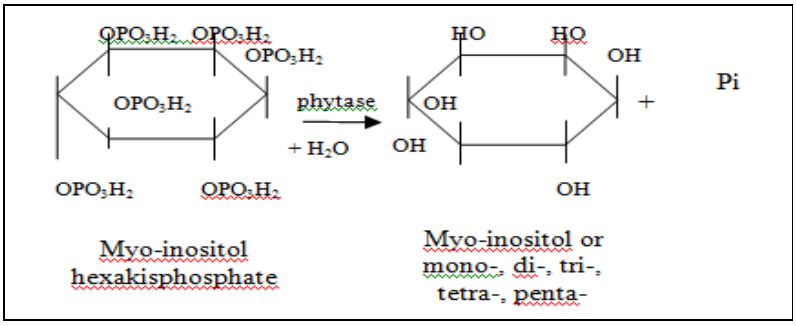

Fig. 1: Dephosphorylation of *myo*-inositol hexakisphosphate (phytate) by phytase.

Rice bran, the brown part of rice kernel, is a by-product of rice milling process, composed of seed coat, the major part of the germ, and most of the outer layer of the kernel together with some broken kernels [5]. Due to highly nutritious content of fat, protein and vitamins, it has been used as animal feed in most part of the world. The bran constitutes about 8.5% of the whole grain [6]. Current research interest is focused in utilizing this rice bran in many areas such as food, environmental, health and industrial purposes. In addition, rice bran also has been used as fermentation substrate for the production of enzymes such as lipase by *Candida* sp. [7], in combination with cassava starch and rice hulls for the production of glucoamylase by *Aspergillus* sp. [8], with wheat bran for the production of alkaline protease by *Trichoderma koningii* [9] and also for the production of protease from *Rhizopus* sp. [10].

The production of *myo*-inositol phosphate intermediates has received much attention due to the remarkable potential health benefits offered by the compounds [11]. *Myo*-inositol phosphates have potential applications in many fields including pharmaceutical research and development. *Myo*-inositol (1,3,4,5,6) pentakisphosphate has an antiangiogenic and antitumour effects which are useful for anticancer therapeutic strategies [12]. Some *myo*-inositol phosphates, including phytate, are present as intracellular molecules [13]. In addition, the second messenger D-*myo*-inositol (1,4,5) trisphosphate has a range of cellular functions including secretion, contraction, cell division, cell differentiation and cell death cell via mobilizing intracellular Ca^{2+} [13]. Meanwhile, it is believed that D-*myo*-inositol (1,2,6) trisphosphate can prevent diabetes complications and treat chronic inflammations besides cardiovascular diseases [14]. Furthermore, a study showed that a combination of inositol hexakisphosphate (IP_6) and

inositol gave better inhibition in different cancers such as in soft tissue, colon, metastaticlung, and mammary cancers than was either one alone [15]. In this present work, anion-exchange chromatography was used to partially separate *myo*-inositol phosphates from the reaction mixturecontaining rice bran and ASUIA279 phytase. The partially purified *myo*-inositol phosphates were then subjected to a cytotoxicity test on MCF-7 cells.

2. MATERIALS AND METHOD

2.1 Production of ASUIA279 Phytase

The cells of ASUIA279 [16] were grown in rice bran with distilled water under aseptic condition (pH 7, 37°C, 250 rpm, 60 hour incubation time). Samples were withdrawn at time intervals, centrifuged at 12,000 rpm for 30 min, 16°C to remove the particulates and the supernatant kept at -20°C for further analysis. Twenty milliliters of aliquot were collected and kept at -20°C for storage.

2.2 Phytase Assay

Phytase assay was performed according to [17] with minor modifications to measure the enzyme activity of phytase produced. It was measured in a mixture of 25 µl of 0.1 M sodium acetate buffer, pH 4.5 and 10 µl 1.03 mM sodium phytate in 200 µL microcentrifuge tube. Enzyme (5 µl) was added to the mixtures to initiate reaction. The mixtures of the enzyme and buffer was incubated for 30 min at 50°C in block heater (SHT 100D, Stuart Scientific). The released phosphate was quantified by ammonium molybdate method [18]. Stop solution (150 µl) consists of acetone / 5N sulphuric acid / ammonium molybdate (2:1:1) was added to the assay mixture followed by 10 µl 1.0M citric acid. Any cloudiness was removed by centrifugation (MiniSpin®, Eppendorf) at room temperature, 10, 000 rpm for 10 min prior to spectrophotometric measurement at 355 nm by micro plate reader, Infinite® 200 Pro series (TECAN Group Ltd.). The phytase activity was expressed as 1 micromol of phosphate liberated per min (Equation 3.1). ($\varepsilon_{microplate}$ = 0.3094 µ / M. cm; $d_{depth\ of\ microplate\ for\ 200\ \mu l\ solution}$ = 0.59 cm; t = incubation time (min); Abs_{355} = Absorbance at 355 nm; V_{total} = total volume of assay solution; V_{enzyme} = volume of enzyme used).

$$Activity = \frac{Abs_{355} \times V_{total}}{d \times \varepsilon \times t \times V_{enzyme}} \quad (1)$$

2.3 Extraction of *Myo*-inositol Phosphates

Rice bran (donated by BERNAS Tanjung Karang, Selangor Darul Ehsan) was used as a fermentation media for the production of bacterial phytase from local isolates, ASUIA279. The cell culture broth of ASUIA279 was grown in the presence of 10% w/v rice bran with distilled water under aseptic condition and pH 7, 37°C and 250 rpm. Samples withdrawn from 0 hour until 72 hours were then centrifuged at 12,000 rpm for 30 min to remove particulates and the supernatant kept at -20°C for further analysis.

2.4 Separation and Recovery of *myo*-inositol Phosphates

AG1 X-4 (100-200 mesh) (BioRad Laboratories, USA) anion exchanger resin was packed in a glass column (2.5 x 30 cm) and equilibrated with distilled water. About 15 ml of the supernatant was loaded on to the column. Three concentrations of hydrochloric acid (Merck, Germany) were used to elute the sample in a stepwise gradient; 0.1 M, 0.5 M, 1.0

M. All collected samples were kept at 4°C. Following thawing, solvent was removed by a rotary evaporator. The dried samples were diluted with a small amount of distilled water and further concentrated by a spin-concentrator. The completely dried residue (partially purified myo-inositol phosphate) was kept in a desiccator to avoid any reintroduction of moisture. The percentage yield of *myo*-inositol phosphates and *myo*-inositol phosphate intermediates eluted by three different concentration of HCl from ASUIA279were determined.

$$yield\,(\%) = \frac{w2 - w1}{w1} \times 100 \quad (2)$$

w1 = weight of centrifuge tube

w2 = weight of centrifuge tube and dried sample

2.5 Effects of *Myo*-inositol Phosphates on MCF-7 Breast Cancer Cell Line

2.5.1 Preparation of Standards and Samples for MCF-7 Tests

Six standard samples of *myo*-inositol phosphates were used as reference during the investigation (Table 1). The standards were diluted in 1ml of 10% DMSO.

Table 1: Amount of *myo*-inositol phosphates standards used.

Standard	**Concentration (Stock solution) (g/ml)**	**Concentration (Dilution) (µg/ml)**
IP_1	0.05	1000
IP_2	0.0005	100
IP_3	0.0025	500
IP_4	5×10^{-5}	10
IP_5	2.5×10^{-4}	50
IP_6	0.125	1000

A small quantity of partially purified *myo*-inositol phosphates were dissolved in 1ml of 10% DMSO to a maximum concentration (Table 2). MCF-7 cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and the cell lines were maintained at 37˚C in a 5% CO_2 incubator and the media were changed twice weekly.

2.5.2 Cancer Inhibition Assay: Sulforhodamine B (SRB) Assay

The SRB assay was performed to assess growth inhibition based on colorimetric principles. This assay estimates cell number indirectly by staining total cellular protein with SRB dye. Briefly, 190 µl/well of cell suspensions (seeding density of 2.0×10^4 cells per well) and 10 µl/well *myo*-inositol phosphates of different concentrations (31.25 µg/ml to 1000 µg/ml prepared in DMSO, Table 2) were seeded in 96-well microtiter plate. The 96-well plate was then incubated for 72 hours at 37ºC and 5% CO_2, to allow for cell attachment. Without removing the cell culture supernatant, the cells were fixed with 100 µl of 10% (w/v) trichloroacetic acid at 4˚C for 1 hour and the supernatant was removed from the 96 well plates. The plate was then washed four times with slow running tap water

and excess water was removed using a blow dryer or just allowed to stand at room temperature. Each well was stained for 30 min with 100 µl of 0.4% (w/v) SRB dissolved in 1% acetic acid and washed four times with 1% acetic acid. The protein bound dye was solubilized with 200 µl of 10 mmol/l Tris base, pH 10.5. The absorbance (OD) of each well was read on an ELISA plate reader at 510 nm. Percentage of control cell growth was calculated using the formula: % of cell growth = [mean OD sample/mean OD negative control]. A dose response curve was then plotted. Negative control = 10% DMSO.

Table 2: Concentration of *myo*-inositol phosphates in DMSO.

Sample aliquot (hour)	**Eluent (HCl) concentration (Molar, M)**	**Weight of *myo*-inositol phosphates (µg)**	**Concentration of *myo*-inositol phosphates in 1 ml 10% DMSO (µg/ml)**
Before inoculation	0.1	2500	2500
	0.5	4000	4000
	1.0	2800	2800
0	0.1	6100	6100
	0.5	4600	4600
	1.0	2600	2600
16	0.1	5800	5800
	0.5	40300	40300
	1.0	66800	66800
24	0.1	24400	24400
	0.5	7700	7700
	1.0	3900	3900
40	0.1	13000	13000
	0.5	5700	5700
	1.0	5500	5500
48	0.1	18300	18300
	0.5	5000	5000
	1.0	2300	2300
64	0.1	14300	14300
	0.5	1500	1500
	1.0	4400	4400
72	0.1	5600	5600
	0.5	9400	9400
	1.0	2500	2500

3. RESULTS AND DISCUSSION

3.1 Analysis of *Myo*-inositol Phosphate Obtained

Three different concentration of eluent: 0.1 M, 0.5 M and 1.0 M HCl (Table 3) were used to elute the *myo*-inositol phosphates from AG1 X-4 resins. Further, ASUIA279 was incubated and monitored for 72 hours. The highest yield of *myo*-inositol phosphates obtained is at 16 hours incubation time (Fig.2) and then it started to decrease by time. It is known that during enzymatic phytate degradation, the hydrolysis rate decreased markedly [19]. This might be due to product inhibition by phosphate or a lower hydrolysis rate of the lower phosphate esters of *myo*-inositol. Another factor is because of the conditions used during the hydrolysis reaction [20]. A very slow degradation also had been observed by Van der Kaay and Van Haastert [21] which is during degradation of *myo*-inositol trisphosphate intermediate and the missing capability to degrade the *myo*-inositol bisphosphate intermediate.

Table 3: Percentage yield of *myo*-inositol phosphates and intermediates eluted by three different concentration of HCl from ASUIA279.

Sample aliquot (hour)	**Eluent (HCl in M)**	**Weight of *myo*-inositol and its intermediates (μg)**	**Yield (%)**
Before inoculation (control)	0.1	0.0811	7.68
	0.5	0.0495	4.69
	1.0	0.045	4.26
0	0.1	0.1126	10.67
	0.5	0.049	4.64
	1.0	0.0516	4.89
16	0.1	0.0788	7.47
	0.5	0.2009	19.03
	1.0	0.02991	28.34
24	0.1	0.0681	6.45
	0.5	0.0188	1.78
	1.0	0.0111	1.056
40	0.1	0.1046	9.91
	0.5	n.a*	n.a*
	1.0	0.0521	4.94
48	0.1	0.0785	7.44
	0.5	0.0441	4.18
	1.0	0.0047	0.45
64	0.1	0.1291	12.23
	0.5	0.0435	4.12
	1.0	0.0414	3.92
72	0.1	0.0546	5.17
	0.5	0.149	14.17
	1.0	0.0438	4.15

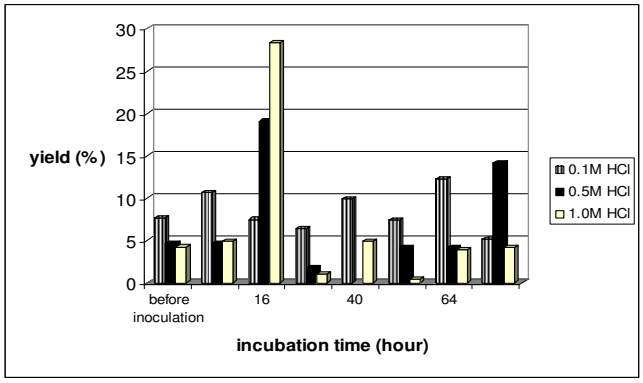

Fig. 2: Fraction of *myo*-inositol phosphate intermediates separated by anion exchange chromatography from ASUIA279 by three different concentrations of HCl.

Since lower *myo*-inositol phosphates have less negative charge compared to higher *myo*-inositol phosphates, the lower *myo*-inositol phosphates (e.g. IP_1, IP_2, IP_3) will be eluted earlier and easily by low concentration of HCl. Meanwhile, higher *myo*-inositol phosphates (e.g. IP_4, IP_5, IP_6) will be eluted by higher concentration of HCl. Moreover, the effective charges of *myo*-inositol phosphates (e.g. IP_5 and IP_6) will be decreased if the acidity of eluent is increasing, which proves the existence of ion suppression mechanism in eluent [22]. The higher the effective charge of the analyte, the longer the retention time of the IPs, and this is also applicable to the isomers. The fluctuated reading of the yield might be caused by the insufficient elution of the *myo*-inositol phosphates or weaker binding of the phytate to the anion exchanger resin [23].

3.2 The Effects of *Myo*-inositol Phosphates to MCF-7 Breast Cancer Cell Line

A novel anticancer function of inositol hexaphosphate (IP_6) has been shown both *in vivo* and *in vitro* [24]. Commonly, most research about inhibition of cancer cells growth were focused on IP_6 and applied in various cell lines including human leukemia cells [25], human colon cancer cells [26], both estrogen receptor-positive and estrogen receptor-negative human breast cancer cells [27], laryngeal carcinoma [28], cervical cancer [29], prostate cancer [30], hepatoma [31], pancreatic [32] and melanoma cell line [33].

Due to a limited amount of standards available, different concentration of sample was used. As can be observed from the experiment, multi well plate that contain IP_6 and IP_1 immediately change to yellow colour after exposure of cell to the solution containing *myo*-inositol phosphates. In consequence, highest concentration of IP_1 and IP_6 showed good inhibition towards the MCF-7 cell line (Fig. 3). This showed that IP_6 supported by Vucenik and Shamsuddin [15] , and IP_1 efficiently inhibit the cancer cell growth. Based on the observation, minimum concentration of *myo*-inositol phosphates (<1000 µg/ml) contributed minimum inhibition against the growth of MCF-7 cell line.

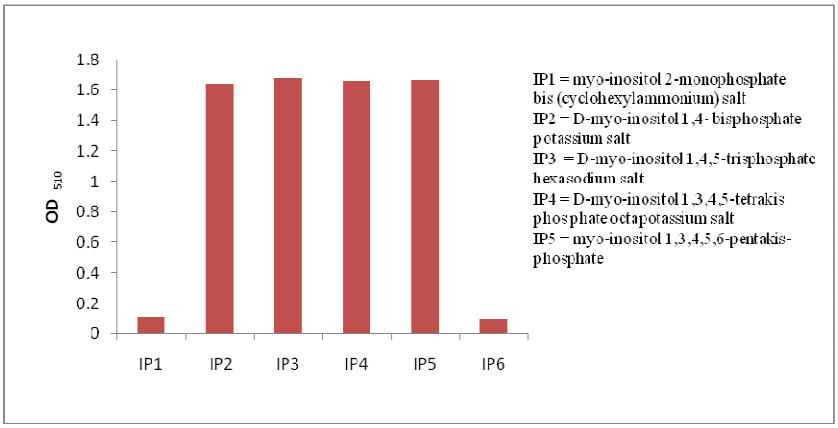

Fig. 3: Optical density of six standards of *myo*-inositol phosphates measured at 510 nm.

The next step is to study the inhibition of partially purified *myo*-inositol phosphates extracted from rice bran towards the MCF-7 cell line. Table 2 listed the concentration of *myo*-inositol phosphates used in this study. Prior to that, a serial dilution of samples ranges from 62.5 µg/ml to 1000 µg/ml were applied to MCF-7 cell line. Nevertheless, no significant changes had been observed throughout the experiment. Once again, lower concentration (<1000 µg/ml) of partially purified *myo*-inositol phosphates were not capable to inhibit the growth of MCF-7 cell line. Since rice bran only has 4% of phytate content [34] compared to pure phytate, it is relevant if 1000 µg/ml was not efficiently inhibit the growth of MCF-7 cells as the standard of IP_1 and IP_6. Theoretically, in 1ml of sample, about 0.04 fractions were *myo*-inositol phosphates. The insufficient amount of *myo*-inositol phosphates used before, leads us to use larger amount of *myo*-inositol phosphates which diluted in 10% DMSO (Table 2).

The SRB assay was then carried out and measured by microplate reader at 510 nm. Figure 4 illustrated the percentage of growth for MCF-7 cells after applied by partially purified *myo*-inositol phosphates. The minimum amount of observed optical density indicated the inhibition of MCF-7 cells growth in the mixture. Two samples showed positive results supporting this conclusion. A decrement of growth had been observed for samples at 16 hour incubation time for 0.5M and 1.0M elution of HCl respectively. The highest concentration contained in both samples proved that the growth of MCF-7 cells really can inhibit by partially purified *myo*-inositol phosphates. According to statement by Adachi et al. [34], about 1.612 mg/ml and 2.672 mg/ml of phytates content in both samples, respectively. However, for samples containing less than 30mg/ml of partially purified *myo*-inositol phosphates showed none or minimum inhibition of MCF-7 cells growth.

Moreover, throughout the analysis, more than 40% inhibition of MCF-7 cells had been observed by partially purified *myo*-inositol phosphates extracted from rice bran. About 44.6% and 46.6% of inhibitions had been calculated for both 0.5M and 1.0M elution of HCl at 16 hour incubation time of ASUIA279. Mostly, *myo*-inositol phosphates consist of IP_3, IP_4, IP_5 and IP_6 were in both samples and able to inhibit the MCF-7 cells growth. IP_6 inhibited the growth of cancer cell lines in a dose- and time-dependent manner, irrespective of whether they were epithelial or mesenchymal in origin [35]. Moreover, the anticancer activity of IP_6 is a result of its rapid intake by tumor cells was shown when MCF-7 human breast cancer cells were incubated with [^{3}H]-IP_6. As early as 1 minute after incubation, 3.1% of IP_6-associated radioactivity was taken up by MCF-7 cells

and 9.5% after 1 hour. Anion-exchange chromatography showed that 58% of the absorbed radioactivity was in IP_6 form, indicating that externally applied IP_6 enters the cells followed by dephosphorylation. However, IP_4 appeared to be a predominant metabolite of IP_6, which possibly might have important role in its anticancer activity [35]. Shamsuddin and Vucenik [36] cited that IP_6 also was used to enhance the anti-proliferative effects of tamoxifen and adriamycin to MCF-7 cells line.

As a whole, IP_6 and its metabolites can differentially inhibit the proliferation of cancer cells without affecting the normal cells, inhibit cell proliferation of cancer cells irrespective of their hormonal receptor status and individual IP_6 metabolite(s) or combinations could be specifically effective against specific cancers, thereby increasing the chances of successful therapy [34].

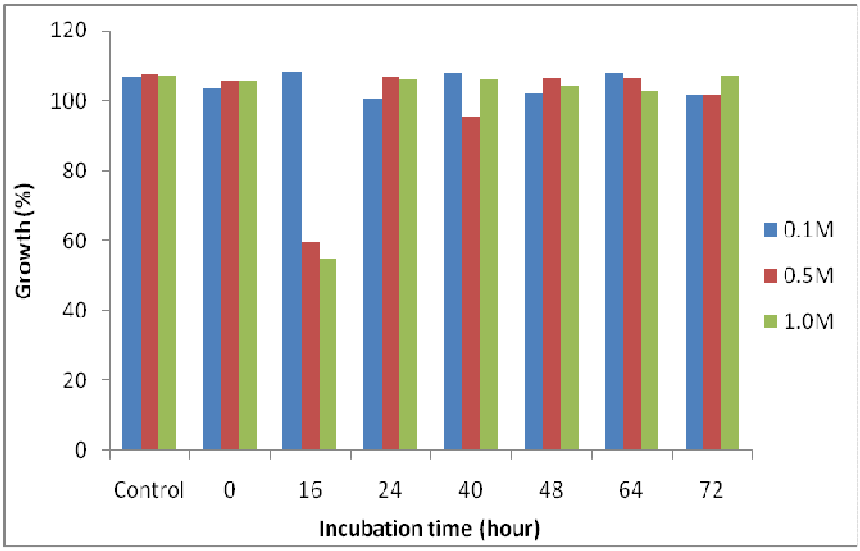

Fig. 4: Percentage of MCF-7 cells growth after treated with partially purified *myo*-inositol phosphates from three different elution concentration of HCl at different incubation time.

4. CONCLUSION

As a conclusion, the application of partially purified rice bran *myo*-inositol phosphates degraded by ASUIA279 to MCF-7 breast cancer cells exhibit positive results towards the inhibition of cancer cells growth with more than 40%. Therefore, through this research findings, a promising value-added product can be obtained from low cost and easily available raw material, rice bran, via hydrolysis reaction by phytase.

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