Iranian Biomedical Journal 12 (3): 167-172 (July 2008)

Pectic Acid Effects on Prolactin Secretion in GH3/B6 Rat Pituitary Cell Line

Delaram Eslimi[1 & 2], Houri Sepehri[1], Yasaman Rassouli[1], Samideh Khoei[3] and Bahram Goliaei[*2]

[1]School of Biology, University College of Science, University of Tehran, Tehran; [2]Institute of Biochemistry and Biophysics, University of Tehran, Tehran; [3] Dept. of Medical Physics, School of Basic Science, Iran University of Medical Science, Tehran, Iran

Received 5 April 2007; revised 9 September 2007; accepted 10 October 2007

ABSTRACT

Background: Pectic acid extracted from plants increases the secretion of prolactin (PRL) when injected intravenously into ewes or fed to rats. Fragments of ewe hypophysis and lactating rabbit mammary gland incubated *in vitro* in the presence of pectic acid secreted more PRL and caseins compared to the controls. However, it is not known whether pectic acid directly stimulates PRL secretion in pituitary or interference of factors from hypophysis is required for this process. **Methods:** GH3/B6 cells, a clonal strain of rat pituitary, were cultured and incubated with pectic acid (2.5-100 μg/mL). The integrity of cells was examined under pectic acid treatment microscopically. Controls or pectic acid treated cells were assayed for their ability to produce PRL. The PRL was assayed by Western-blotting and Radioimmunoassay. **Results:** pectic acid did not have any significant effect on the viability of cells. After being incubated with pectic acid, the cells started to become circular and protuberant shape. The maximum stimulation and PRL secretion occurred at 100 μg/mL concentration within 30 min of incubation with pectic acid. **Conclusion:** pectic acid could stimulate the release of PRL in GH3/B6 cells in the short-term incubation. This result suggested that pectic acid is a non-toxic agent that could directly stimulate PRL secretion in pituitary cells without any interference of hypophysis. *Iran. Biomed. J. 12 (3): 167-172, 2008*

Keywords: Pectic acid, Prolactin, Pituitary, GH3/B6 cells

INTRODUCTION

There is a continuing interest in identification and better understanding of lactogenic plants and their extracts [1]. Pectins from apple, citrus and sugar beet, pectic acid, and polygalacturonic acid injected intravenously to ewes markedly stimulated blood prolactin (PRL), growth hormone (GH) and cortisol [2].

Pectic acid was administered orally to mature virgin rats induced the accumulation of beta-casein in mammary gland [3]. Pure beta-glucan extracted from barley also stimulated hormone secretion when it is administered by the intravenous route [4].

It has been shown that beta-glucan and several pectin derivatives were able to stimulate PRL secretion from hypophysis fragments incubated in a synthetic medium for 2 h [5, 6]. *In vitro* studies have shown that extracts from lactogenic plants induce β-casein synthesis in epithelial cells of rat mammary glands [7].

The mechanism of actions of pectins on PRL production by anterior pituitary cells is not well understood. Pectin has a linear backbone composed of units of (1, 4)-linked α-D-galacturonic acid and its methyl ester units interspersed to some degree with L-rhamnose, L-galactose and L-arabinose. The galacturonic acid units may be in the salt form 'galacturonate' which makes pectin an anionic polymer. Completely de-esterified pectin is termed pectic acid or pectate [8].

In this study, we have used GH3/B6 cells, a rat pituitary tumor cell line, which synthesizes and secretes large amounts of PRL *in vitro*. It has been

*Corresponding Author; Tel. (+98-21) 6649 8672; Fax: (+98-21) 6695 6985; E-mail: goliaei@ibb.ut.ac.ir

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2008.12.3.1.8]

[DOI: -]

shown that 50-70% of the cells content of PRL is concentrated in the Golgi region [9-11]. We demonstrate that pectic acid can directly stimulate the release of PRL from GH3/B6 cells without interference of factors from hypophysis

MATERIALS AND METHODS

Cell culture. GH3/B6 cells, subcloned from GH_3 tumor-derived rat pituitary cells, was a kind gift by Dr. Gourdji [12]. These cells were routinely grown as monolayer in serum-supplemented medium (SSM) in Ham's F-12 medium enriched with 15% heat-inactivated horse serum (Gibco®, USA) and 2.5% fetal calf serum (Gibco®, USA) and with antibiotics (50 mU/ml of penicillin plus 50 μg/ml of streptomycin or 40 μg/ml of gentamicin) in a humidified atmosphere of 5% CO_2 at 37˚C [13]. Cultures were propagated by trypsinization with 1 mM EDTA/0.25% trypsin (w/v) in PBS.

Cell survival determination. The viability of the cells was determined by trypan blue dye exclusion test. Penetration of trypan blue dye was used as an indicator of viability of the cultured cells, based on the principle that live cells exclude the dye.

Pectic acid treatment. CH3/B6 cells were seeded at 5×10^4 cells/ml in SSM medium in 24-well plates. After 3 days, the culture medium was replaced with fresh SSM medium, then stimulated with pectic acid at final concentrations of 0, 2.5, 100 μg/mL for 30 min, 48 h, and 72 h [6]. The conditioned media were collected for further analysis.

HEPES treatments. To study the effects of HEPES on GH3/B6 cells, Ham's F12 culture medium with HEPES (25 mM) was used (Himedia, India). Morphology and viability of cells cultured with this medium were evaluated.

Radioimmunoassay (RIA). PRL was measured in the culture medium by RIA in triplicate sample. The hormone concentration was measured using NIDDK-rPRL-RP-3 (The National Institute of Diabetes and Digestive and Kidney Diseases**)** reference and a specific anti-rat PRL antibody [14].

Western-blot analysis. PRL detection in conditioned media in the presence or absence of pectic acid was performed by western blotting. Briefly, GH3/B6 cells were cultured at 1×10^6 cells in 60 mm Petri dishes (NUNC, Denmark). The cells were stimulated with 2.5 or 100 μg/mL of pectic acid for 30 min. Equal amounts of culture medium were mixed with an equal volume of SDS-PAGE loading buffer, heated at 90°C for 5 min. Then, conditioned media were analyzed
by SDS-PAGE and transferred to nitrocellulose membrane (Schleider and Schuell, Germany).

Membranes were blocked with BSA at room temperature for 1 h followed by incubation with polyclonal rabbit anti-rat PRL (Oxford Biotechnology, UK) diluted 1:300 in Tris buffered saline with 0.05% Tween-20 (TBS/Tween) overnight. Then, incubated for 1 h at room temperature with anti-rabbit IgG conjugated with horse radish peroxidase diluted 1: 12500 in TBS/Tween to generate a 4- Chloro 1- Naphtol signal (Sigma-Aldrich, Germany).

For identification, protein standard of PRL was included in the 12% SDS-PAGE. The protein standard was visible on the nitrocellulose after Western-blot. The antibody labeled PRL band on the developed blots was digitally captured with an ultra violet imager Tec and band intensities were measured using Totallab® software (image analysis software). In parallel, GH3/B6 cells were stimulated with thyrotropin releasing hormone (TRH) at final concentration of 50 nM as positive control.

Statistical analysis. The data are expressed as means ± SEM. The Statistical analyses were performed using one-way analysis of variance (ANOVA). Following a significant F-value, post-hoc analysis (Tukey) was performed to assess specific group comparisons. Student's *t*-test used to compare control versus different concentrations of pectic acid. Significant differences was defined as $P<0.05$.

RESULTS

Effect of HEPES on morphology of GH3/B6 cell lines. The cells cultured in Ham's F12 culture medium with HEPES (Fig. 1A) compared to without HEPES (Fig. 1B), developed cytoplasmic vacuoles and membrane inclusion bodies. There was a reduction in viability of the cells from 97 ± 2.5% without HEPES to 81 ± 1.5% with HEPES (mean ± SE).

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2008.12.3.1.8]
[DOI: -]

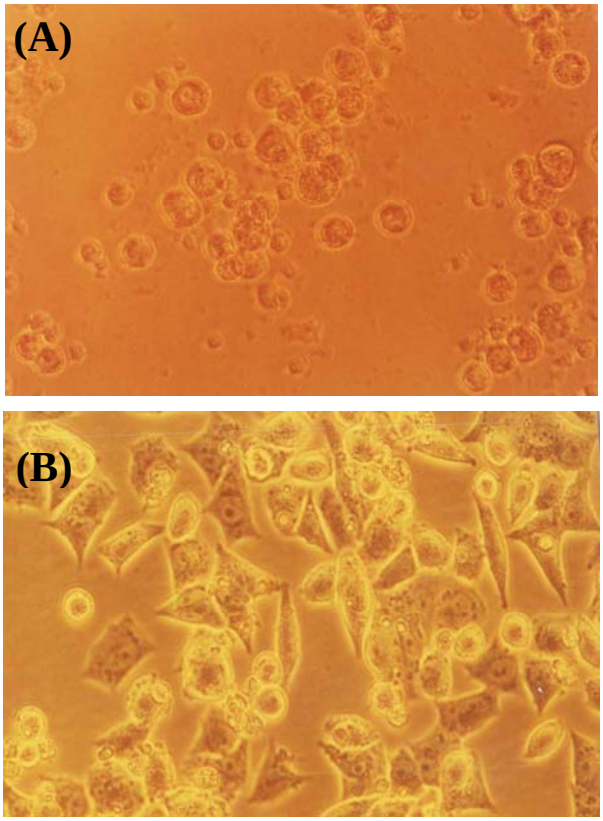


Fig. 1. Effects of HEPES on GH3/B6 cells morphology. **(A)** Culture medium without HEPES and **(B)** Culture medium with HEPES (32 ×).

Effect of pectic acid on morphology and cellular viability of GH3/B6 cell lines. Pectic acid In the presence of pectic acid (2.5 and 100 µg/mL) for 30 min (Figs. 2A and 2B) GH3/B6 cells became spherical and loosely attached to tissue culture surface compared to controls (Fig. 2C). We have observed similar cell morphology in cells stimulated with TRH as positive control (Fig. 2D).

Cellular viability. The effect of pectic acid and TRH on the viability of GH3/B6 cells was examined by trypan blue dye exclusion test. pectic acid at the concentrations of 2.5 and 100 µg/mL and TRH at the concentration of 50 nM did not have any significant effect on the viability of cells (mean ± SE).

***Effect of pectic acid on PRL level*:**

RIA analysis. PRL secretion following pectic acid treatment in these cells was further studied by measuring the PRL in the samples as described above with RIA.

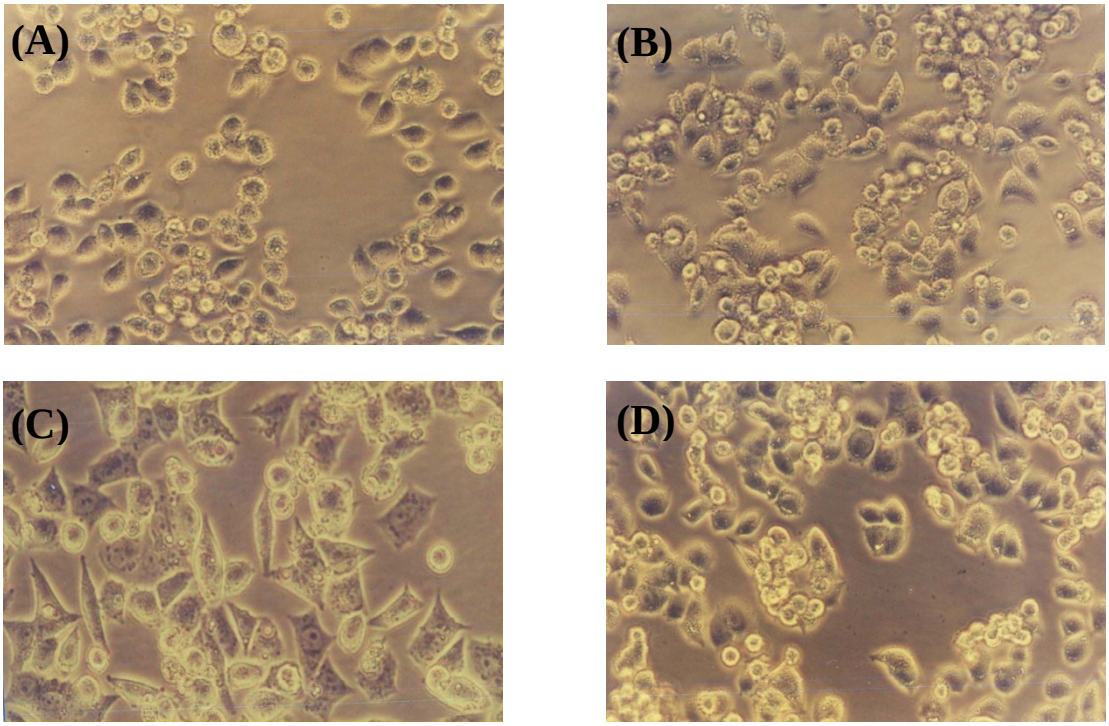


Fig. 2. GH3/B6 cells after 30 minutes of incubation with pectic acid and TRH. **(A)** pectic acid (2.5 µg/mL); **(B)** pectic acid (100 µg/mL); **(C)** Control and **(D)** TRH (50 nM) (32 ×).

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]
[DOR: 20.1001.1.1028852.2008.12.3.1.8]
[DOI: -]

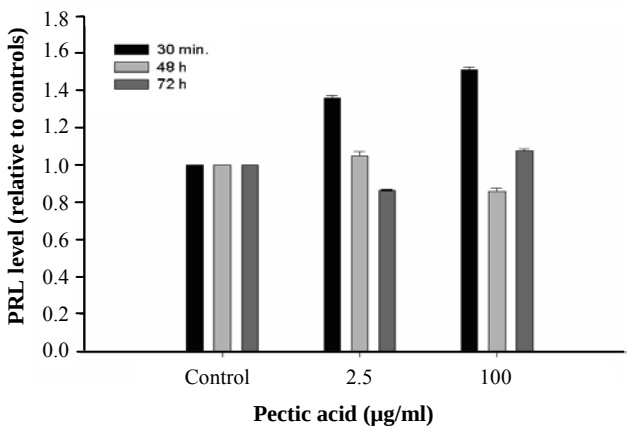


Fig. 3. Effects of pectic acid on PRL secretion by GH3/B6 cells measured with RIA. Cells were incubated for 30 mins, 48 h, and 72 h with various concentrations of pectic acid. The amount of PRL produced by treated samples is compared with respect to their controls. The amount of PRL in the control samples was 47.6 ± 7.1 pg/µg protein/mL. Only samples with 30 mins of incubation with pectic acid had statistically significant differences in PRL values with respect to controls. Mean ± SEM.

As shown in the Figure 3, maximum stimulation of PRL secretion was detected 30 min following incubation of cells with 100 µg/mL of pectic acid. In these samples, the amount of PRL released into the medium increased as a function of pectic acid concentration in the dose range studied. In 30-min samples, the increase in PRL release due to pectic acid at all concentrations studied was statistically significant with respect to controls (mean ± SE) (Fig. 3).

Western-blot analysis. To further illustrate the release of PRL in the culture medium under pectic acid stimulation, the culture media collected after 30 min of pectic acid stimulation were analyzed by Western-blotting. TRH, as positive control, was used for stimulation of PRL release (Fig. 4). Again, maximum stimulation of PRL release was observed at 100 µg/mL of pectic acid (mean ± SE). At all concentrations studied, pectic acid could stimulate the release of PRL better than TRH.

DISCUSSION

Several factors regulate the PRL secretion *in vivo* such as TRH, epidermal growth factor, fibroblast growth factor, galanin, estrogen and others [15-19]. These experiments have indicated that pectic acid, one of the major components of the lactogenic plant, is able to stimulate the secretion of PRL, GH, luteotropin hormone and β-endorphin from the rat hypophysis [2]. Pectin is a major component of primary cell walls of all land plants and encompasses a range of galacturonic acid-rich polysaccharides (pectic acid) [8]. Pectin has multiple beneficial effects on human health and tumor growth control [20, 21].

Our results confirm that pectic acid, similar to other pectins, was non-toxic to cells. Pectic acid can stimulate PRL secretion in short term incubation. This was supported by the alternate morphology of GH3/B6 cells in the presence of pectic acid or TRH. These observations suggest that pectic acid effects are likely exerted directly at the lactotrop level and do not depend on another complex interactions. Also the viability of the cells show pectic acid hasn't any toxically effects on the cells.

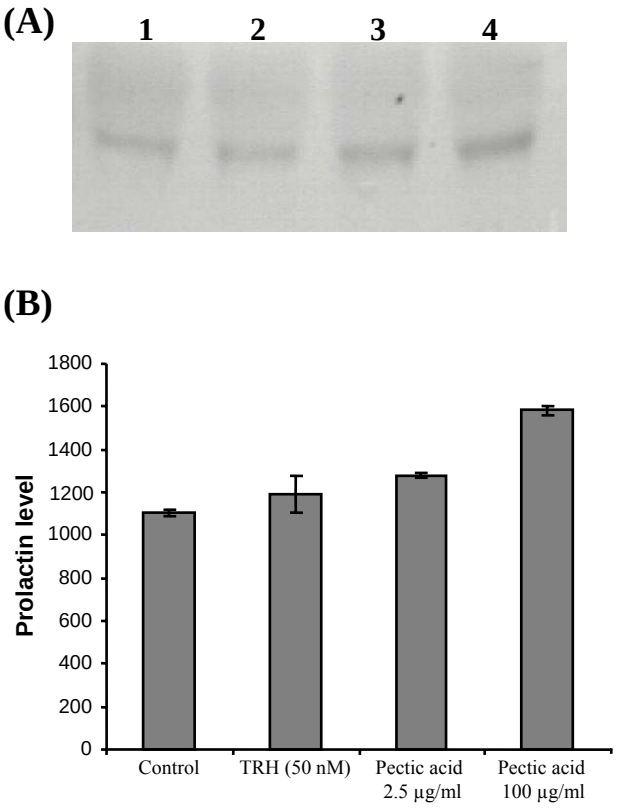


Fig. 4. (A) Western-blot of PRL. Lane 1, Control; lane 2, TRH (50 nM); lane 3, pectic acid (2.5 µg/mL); lane 4, pectic acid (100 µg/mL). **(B)** Results of scanning the bands by Totallab® software (mean ± SEM).

Pectic acid decreases cell culture medium pH and this is not suitable for the cells. HEPES is used extensively in cell culture due to its excellent buffering capacity but had a toxic effect on GH3/B6

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]
[DOR: 20.1001.1.1028852.2008.12.3.1.8]
[DOI: -]

cells. The toxicity exhibited by HEPES appears to be mediated by the production of reactive oxygen species. The toxicity was implicated extra cellular hydrogen peroxide as the toxic agent. HEPES stimulated the production of toxic oxygen metabolites resulting in a decrease in the growth [22].

It has been reported that pectic acid is a direct precursors of substances named called elicitors. Elicitors are fragments of pectins and β-glucan which recognize specific receptors on plant cells. Elicitors act as vegetal hormones by inducing the expression of certain genes and defensive responses [23-25]. No receptor has been identified in cells of higher vertebrates for pectic acid. A possible hypothesis has been forwarded that pectic substances have some structural homologies with extra cellular matrix of mammalian cells and the active compounds of plant extracts might affect cellular secretion by binding to these receptors [18]. It remains to identify cellular receptors involved and by which mechanism the stimulatory effects of active compounds of plant extracts on PRL secretion are exerted. In this paper, the direct stimulation of PRL secretion by pectic acid without any interference from pituitary factors in GH3/B6 rat pituitary tumor cells was demonstrated.

ACKNOWLEDGMENTS

We thank Dr. Ghamar Taj Hossein for helpful suggestions on manuscript preparation.

REFERENCES

1. Lompo-Ouedraogo, Z., Van der, H.D., Van der Beek, E.M., Swarts, H.J., Mattheij, J.A. and Sawadogo, L. (2004) Effect of aqueous extract of *Acacia nilotica* ssp. adansonii on milk production and prolactin release in the rat. *J. Endocrinol. 182: 257-266.*
2. Sawadogo, L., Houdebine, L.M., Thibault, J.F., Rouau, X. and Ollivier-Bousquet, M. (1988) [b] Effect of pectic substances on prolactin and growth hormone secretion in the ewe and on the induction of casein synthesis in the rat. *Reprod. Nutr. Dev. 28: 293-301.*
3. Sawadogo, L. and Houdebine, L.M. (1988) [a] Induction of beta-casein synthesis in the mammary glands of rats treated with plant extracts. *C.R. Acad. Sci. III. 306: 167-172.*
4. Sawadogo, L., Sepehri, H. and Houdebine, L.M. (1989) Evidence for a stimulating factor of prolactin and growth hormone secretion present in brewery draff. *Reprod. Nutr. Dev. 29: 139-146.*
5. Sepehri, H., Renard, C. and Houdebine, L.M. (1990) Beta-glucan and pectin derivatives stimulate prolactin secretion from hypophysis *in vitro*. *Proc. Soc. Exp. Biol. Med. 194: 193-197.*
6. Sepehri, H., Zoraghi, R. and Haeri Rouhani, A. (2000) Effect of pectic acid and β-glucan on prolactin secretion by ovine pituitary explants. *Iran. Int. J. Sci. 1(2): 99-109.*
7. Sawagado, L., Houdebine, L.M., Thibault, J.F. and Rouau, X. (1988) Mise en evidence d'une actvie lactogene dan des extraits vegetaux. *Bull. Med. Trad. 2: 19-30.*
8. Willats, W.G., McCartney, L., Mackie, W. and Knox, J.P. (2001) Pectin: cell biology and prospects for functional analysis. *Plant Mol. Biol. 47: 9-27.*
9. Hoyt, R.F. Jr. and Tashjian, A.H. Jr. (1980) Immunocytochemical analysis of prolactin production by monolayer cultures of GH3 rat anterior pituitary tumor cells: I. Long-term effects of stimulation with thyrotropin-releasing hormone (TRH). *Anat. Rec. 197: 153-162.*
10. Ooi, G.T., Tawadros, N. and Escalona, R.M. (2004) Pituitary cell lines and their endocrine applications. *Mol. Cell Endocrinol. 228: 1-21.*
11. Tashjian, A.H. Jr. (1979) Clonal strains of hormone-producing pituitary cells. *Methods Enzymol. 58: 527-535.*
12. Gourdji, D., Laverriere, J.N., Passegue, E. and Richard, J.L. (1992) Hypophyseal cells model systems: the "GH" rat tumor-derived cell lines as a tool for the study of gene expression. *Cell Biol. Toxicol. 8: 29-38.*
13. Gourdji, D. and Tixier-Vidal, A. (1980) Prolactin secreting cell lines: a tool for the study of the mechanism of action of hypophysiotropic neuropeptides. *A review (author's transl)]. J. Physiol. (Paris) 76: 233-241.*
14. Vila-Porcile, E., Picart, R., Olivier, L., Tixier-Vidal, A. and Tougard, C. (1988) Subcellular distribution of laminin and prolactin in stimulated and blocked prolactin cells in the pituitary of lactating rats. *Cell Tissue Res. 254: 617-627.*
15. Heaney, A.P., Fernando, M. and Melmed, S. (2002) Functional role of estrogen in pituitary tumor pathogenesis. *J. Clin. Invest. 109 (2): 277-283.*
16. Heaney, A.P. and Melmed, S. (2004) Molecular targets in pituitary tumours. *Natl. Rev. Cancer 4 (4): 285-295.*
17. Mallo, F., Wilson, E., Whorwood, C.B., Singh, S. and Sheppard, M.C. (1995) Basic and acidic fibroblast growth factor increase prolactin mRNA in a dose-dependent and specific manner in GH3 cells. *Mol. Cell Endocrinol. 114(1-2): 117-125.*
18. de Carvalho, D.F., Silva, K.L., de Oliveria, D.A., Villa-Verde, D.M., Coelho, H.S., Silva L.C. and Nasciutti, L.E. (2000) Characterization and

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]
[DOR: 20.1001.1.1028852.2008.12.3.1.8]
[DOI: -]

distribution of extracellular matrix components and receptors in GH3B6 prolactin cells. *Biol. Cell 92: 351-362.*

19. Ray, K.P. and Wallis, M. (1984) Studies of TRH-induced prolactin secretion and its inhibition by dopamine, using ovine pituitary cells. *Mol. Cell Endocrinol. 36: 131-139.*
20. Hayashi, A., Gillen, A.C. and Lott, J.R. (2000) Effects of daily oral administration of quercetin chalcone and modified citrus pectin on implanted colon-25 tumor growth in BALB/c mice. *Altern. Med. Rev. 5: 546-552.*
21. Salomov, I.T., Ashurov, A.R., Oblakulov, K., Salomov, U.I. and Dzhalilov, B.M. (1994) Effect of pectin additives from cotton on the physical development, nutrition and erythrocytes in infants. *Vopr. Pitan. 6: 16-18.*
22. Bowman, C.M., Berger, E.M., Butler, E.N., Toth, K.M. and Repine, J.E. (1985) HEPES may stimulate cultured endothelial cells to make growth-retarding oxygen metabolites. *In vitro Cell Dev. Biol. 21: 140-142.*
23. Gupta, A.K. and Kaur, N. (2005) Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *J. Biosci. 30: 761-776.*
24. Prome, J.C. (1996) Signalling events elicited in plants by defined oligosaccharide structures. *Curr. Opin. Struct. Biol. 6: 671-678.*
25. Ryan, C.A. (1987) Oligosaccharide signalling in plants. *Annu. Rev. Cell Biol. 3: 295-317.*

[Downloaded from ibj.pasteur.ac.ir on 2024-03-29]

[DOR: 20.1001.1.1028852.2008.12.3.1.8]

[DOI: -]