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Investigation of aromotase inhibition by several dietary vegetables in human non–small cell lung cancer cell lines

[İnsan küçük hücreli olmayan akciğer kanser hücrelerinde çeşitli gıdalar tarafından aromataz inhibisyonunun araştırılması]

Gurbet Celik[1],
Hakan Akca[2],
Alaattin Sen[1]

Pamukkale University, Kinikli Campus,
[1]Department of Biology, [2]Medical School, 20070
Denizli TURKEY

Yazışma Adresi
[Correspondence Address]

Prof. Dr. Alaattin Sen:

Faculty of Art & Sciences, Biology Department,
Pamukkale University, Kinikli Campus. Kinikli,
Denizli, 20070, Turkey.
Tel. +90-258-2963574
Fax. +90-258-2963535
E-mail. sena@pau.edu.tr

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ABSTRACT

Objective: In this study we have investigated the effect of extracts obtained from well-known vegetable diets consumed commonly in Mediterranean and Aegean regions of Turkey on the expression of aromatase at protein, mRNA and activity levels in human non–small cell lung cancer cells- PC-3 and PC-14 cell lines.

Method: For this purpose, cytotoxicity was determined by using crystal violet stain. *In vivo* and *in vitro* aromatase activity was determined flourometrically. The expression of aromatase in protein and mRNA levels was detected by Western blotting using anti-hCYP19 and reverse transcriptase- polymerase chain reaction using suitable primers, respectively.

Result: Extracts obtained from *Laurus nobilis, Mentha piperita, Crocus sativus* reduced aromatase activity 32%, 44%, 36% and 42%, 32%, 56% in PC-14 and PC-3 cell lines, respectively, no significant changes were observed in protein and mRNA levels. Whereas extract obtained from *Allium porrum* decreased aromatase activity and mRNA level only in PC-14 cell lines by 52% and 2,5-fold, respectively, without significant influence on aromatase protein level.

Conclusion: The results obtained in this study have shown that *Laurus nobilis*, *Mentha piperita*, *Crocus sativus* and *Allium porrum* do contain effective aromatase inhibitors. Therefore, further studies investigating the content of these vegetables are necessary to understand the potential role and mechanism of action of these foods in reducing the risk of non-small cell lung carcinomas.

Keywords: Aromatase, Aromatase Inhibitors, Non-small cell lung cancer, *Laurus nobilis*, *Mentha piperita*, *Crocus sativus* and *Allium porrum*.

Conflict of Interest: The authors have declared that there is no conflict of interest.

ÖZET

Amaç: Bu çalışmamızda Akdeniz ve Ege bölgemizde yaygın olarak tüketilen, bitkisel gıdalardan elde edilmiş özütlerin akciğer kanserinin bir tipi olan küçük hücreli olmayan insan akciğer kanseri hücrelerinde (PC-3 ve PC-14) protein, mRNA ve aktivite düzeylerinde aromataz ifadesi üzerine etkilerini araştırdık.

Yöntem: Bu amaçla sitotoksik etki kristal viyole boyası kullanılarak belirlendi. *In vivo* ve *in vitro* aromataz aktivitesi florometrik olarak tayin edildi. Aromataz protein ifadesi anti-hCYP19 antikorları kullanılarak Western blot ile mRNA ifadesi uygun primerler kullanılarak ters transkriptaz polimeraz zincir reaksiyonu yöntemi ile belirlendi.

Bulgular: *Laurus nobilis, Mentha piperita, Crocus sativus'*dan elde edilen özütler PC-14 ve PC-3 hücre hatlarında aromataz aktivitesini sırasıyla %32, %44, %36 ve %42, %32, %56 oranlarında azaltmıştır, protein ve mRNA seviyelerinde anlamlı değişiklikler gözlenmemiştir. *Allium porrum'*dan elde edilen özüt PC-14 hücrelerinde aromataz aktivitesi ve mRNA ifadesini sırasıyla %52 ve 2,5-kat azaltırken, aromataz protein düzeyinde bir değişiklik yapmamış, PC-3 hücrelerinde herhangi bir etki göstermemiştir.

Sonuç: Bu çalışmada elde edilen bulgular, *Laurus nobilis*, *Mentha piperita*, *Crocus sativus* ve *Allium porrum* etkili aromataz inhibitörü içerdiğini göstermektedir Sonuç olarak, bu gıdaların küçük hücreli olmayan insan akciğer kanseri riskini azaltmada potansiyel rolleri ve etki mekanizmalarını anlamak için bu sebzelerin içeriğini araştıran ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Aromataz, Aromataz İnhibitörü, Küçük hücreli olmayan akciğer kanseri, *Laurus nobilis*, *Mentha piperita*, *Crocus sativus* ve *Allium porrum*

Çıkar Çatışması: Yazarlar hiçbir çıkar çatışması bulunmadığını beyan eder.

Introduction

Introduction

Aromatase [EC 1. 14. 14. 1] is the enzyme that catalyzes three consecutive hydroxylation reactions converting C19 androgens into aromatic C18 estrogens in the biosynthesis of estrogens [1, 2]. Estrogens are female sex hormones involved in the development and growth of hormone-dependent tumors and may; therefore, play pivotal roles in breast cancer development [2]. Furthermore, estrogens contribute to differentiation and maturation in normal lung tissue and stimulate growth of lung cancer tissue, especially in non-small cell lung carcinomas (NSCLCs) [3-5]. NSCLC, account for 80% of all lung carcinomas and are composed of heterogeneous groups such as adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma [6]. Estrogens are produced as a result of intratumoral aromatization has been recently shown to play pivotal roles in proliferation of human NSCLCs [7]. Recent studies have shown that estrogens in lung cancer progression may be promoted by aromatase. Aromatase activity appears to be enhanced in particular estrogen-dependent cancer tissue [5, 8]. Therefore, as in breast, aromatase inhibitors may prove to be useful to prevent estrogen synthesis in lung cancer and are used for the treatment of disease. Competitive and mechanism-based inhibitors of aromatase have been clinically exploited in the treatment of estrogen-dependent mammary tumors, but in order to find novel cancer chemopreventive agents from natural products, we have evaluated the potential of well-known vegetable and herbal diets to inhibit aromatase. Therefore, in this study we have investigated the effects of vegetables, regularly consumed in Mediterranean and Aegean regions of Turkey, on the expression of aromatase enzyme in human NSCLC (PC-14 and PC-3) cell lines.

Materials and Methods

Chemicals: The following chemicals were purchased from Sigma-Aldrich Chemical Company (St Louis, Missouri, USA): RPMI, fetal bovine serum (FBS), trypsin, penicillin/streptomycin mixture, anti-rabbit IgG-HRP conjugate, bovine serum albumin (BSA), *N*, *N*-dimethylformamide, bicinchoninic acid (BCA), glycine, β-nicotinamide adenine dinucleotide phosphate, reduce (NADPH), 4-amidinophenylmethylsulfonyl fluoride (APMSF), **ε amino caproic acid (ε-ACA),** potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium dodecyl sulfate (SDS) and 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS). Polyclonal anti-human *CYP19A* was purchased from Santa Cruz Biotechnology, USA. All other chemicals and solvents were obtained from commercial sources at the highest grade of purity available.

Cell culture: The human lung adenocarcinoma cell line PC-3 (JCRB0077) was purchased Japanese Collection of Research Bioresources (Osaka, Japan) and the human lung adenocarcinoma cell line PC-14 (RCB0446) and human breast adenocarcinoma cell line MCF-7 (RCB1904) were purchased from RIKEN Cell Bank (Ibaragi, Japan). We were obtained from Dr. Jun Yokota, National Cancer Center Research Institute, Japan. The cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin mixture in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C and were sub cultured twice a week.

Microwave-assisted extraction: Vegetables and herbs were purchased from a local herbal store in Denizli, Turkey. The plants herbs were powdered in a blender and were extracted with microwave as described by Hong *et al.* [9] and optimized in our laboratory [10]. SHOV (M7017P-A) microwave-oven was used for microwave assisted extractions (MAE). Sample (5 g) was extracted with methanol/water (20:80 v/v, 100 mL), the extractions being carried at 100 W for 20 seconds to avoid over boiling of suspension followed by cooling the flasks on ice for a minute. Above steps were repeated five times. All Vegetable or Herb Extracts (VHE) was filtered through a 0.2 μm nylon syringe filter (Millipore) before further use in experiments.

Cytotoxic activity of VHEs: PC-3 and PC-14 cells were grown in 96-well plates at a density of $1x10^3$ cells/ml culture medium. After 24 h pre-exposure incubation, the cells were treated with varying concentrations (ranging from 1 μg/ml to 200 μg/ml) of VHE. Equal amounts of medium without VHE were added to untreated cells (control). VHE-treated and control cells were incubated for 48 h at 37 °C in humidified 5% CO_2 atmosphere. Following incubation, the medium was replaced by 0.5% crystal-violet (w/v; in 50% methanol) solution. Plates were incubated for 10 min at room temperature, washed with water and adsorbed dye was eluted out with Na-citrate (0·1 M Na-citrate in 50% ethanol, pH 4·2). Absorbance, which was proportional to cell viability, was measured at a wavelength of 600 nm. Cell viability was monitored as the percentage of viable cells compared to control, untreated cells [11].

Preparation of cell lysates: PC-3 and PC-14 cells ($1x10^6$ cells per 75 cm^2 flask) were exposed to VHE and harvested after a 48-h treatment. Cells were then washed twice with (PBS), scraped from culture dishes in lysis buffer (0.1 M phosphate buffer Tris-HCl, pH 7.8, 0.2% Triton X-100, 2 mM EDTA, 0.5 mM APMFS, 0.3 mM **ε-ACA** and 1 mM DTT), and homogenized mechanically by sonication. Total cellular protein concentration was determined by bicinchoninic acid (BCA) assay using BSA as the standard [12].

***In vivo* and *in vitro* aromatase assay using flourometric substrate dibenzylflourescein:** *In vivo* and *in vitro* microsomal *CYP19*-dependent aromatase activities of control and VHE-treated cells were measured using modified BD Gentest's high throughput recombinant *CYP19* enzyme assay using the fluorometric substrate dibenzylfluorescein as described by [13]. All enzyme assays were performed with freshly prepared cell lysates and optimized for the cell culture conditions. Briefly,

the pre-mixture consisting of 100 mM phosphate buffer pH 7.5, 0.2 mM NADPH and 40 µg microsomal protein was added into a tube and pre-incubated at 30 ºC for 5 minutes. Then 20 µM dibenzyl fluorescein (DBF) was added and the reaction mixture incubated at 30 ºC for 1 hour with shaking. At the end of the incubation period, reaction was stopped by the addition of 1N NaOH and centrifuged at 12,000 xg for 15 minutes. The supernatant was transferred to a new tube and incubated at 37 ºC for 2 hours for color development that was read at Cary Eclipse (Varian Ltd., 28 Manor Road, Walton-on-Thames, Surrey KT12 2Qf, England) fluorometer (Ex= 725 nm, Em= 512 nm). Aromatase activity was expressed as pmol of fluorescein per mg of protein per minute. Apart from CYP19, DBF is also a substrate for other cytochromes, such as CYP2C9, CYP2C19, and CYP3A. In order to verify that aromatase was involved in production fluorescein in our work, we used the selective CYP2C9, CYP2C19, and CYP3A inhibitors sulfaphenazole, tranylcypromineas and ketoconazole, respectively, as positive control and inhibitions of control fluorescein production were corrected.

Gel electrophoresis and Western blotting: The cells were treated with IC_{50} concentrations of VHE were grown to 80% confluence in the plates. The cells were washed with PBS and then were lysed in ice-cold RIPA buffer (10 mM Tris-HCl pH7.5 containing 150 mMM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate and protease cocktail). Debris was removed by centrifugation at 12,000 xg for 5 min at 4 °C. Protein samples (100 µg protein) were separated on 8.5% polyacrylamide gels using the discontinuous buffer system of Laemmli (1970) [14].The proteins were electrophoretically transferred onto nitrocellulose membrane with the iBlot™ Dry Blotting System from Invitrogen (20 V, 12 min). After protein transfer, membranes were then blocked with 5% non-fat dry milk in TBST (10 mM Tris-HCl, pH 7.5 containing 100 mM NaCl and 0.1% (v/v) Tween 20) for 1 h, and incubated with rabbit polyclonal IgG *CYP19* or *GAPDH* antibodies (1: 500 dilution in blocking solution; Santa Cruz, CA). The membranes were washed and incubated with the secondary antibody horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in blocking solution; Santa Cruz, CA). Proteins were detected using SuperSignal® West Pico Chemoluminescent Substrate (Pierce, Rockford, IL, USA), and bands were visualized and recorded using GelQuant Image Analysis Software in a DNR LightBIS Pro Image Analysis System (DNR Bio-Imaging Systems Ltd. Jerusalem, Israel). Protein bands were quantified using Scion Image Version Beta 4.0.2 software [15].

RNA Isolation and RT-PCR of *CYP19* and *18S* mRNA: Total RNA was prepared from VHE-treated and control PC-3 and PC-14 cells using PureLink Micro-to-Midi total RNA purification system following the manufacturer's protocol and was quantified directly using spectrophotometer (Eppendorf BioPhotometer Spectrophotometer UV/VIS, El Cajon, California, USA).

For semi-quantitative RT-PCR assays, both the first strand cDNA synthesis and the PCR amplification were performed in a single tube with gene specific primers using SuperScript III one-step RT-PCR kit following the manufacturer's protocol. The primers for *CYP19* were: forward primer 5'-GAA TAT TGG AAG GAT GCA CAG ACT-3' and a reverse primer 5'-GGG TAA AGA TCA TTT CCA GCA TGT-3' ; for *18S* (housekeeping gene) were: forward 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3' and reverse 5'-TCC TTG GAG GCC ATG TGG GCC AT-3'.

2.5 µg of total RNA were reverse transcribed to cDNA at 55 ºC for 30 min and after an initial 2 min denaturing step at 94 ºC, 40 cycles of amplification were performed using a cycle profile of 94 ºC for 30 s, 61 ºC for 1 min and 68 ºC for 1 min. After the last cycle, elongation was extended to 5 min at 68 ºC. The amplified products were separated on a 1% agarose gel containing ethidium bromide. The intensity of bands was analysed by DNR LightBIS Pro Image Analysis System. Level of mRNA for *CYP19* is expressed relative to *18S* by measuring the band intensity of RT-PCR product on each agarose gel.

Statistical analysis: Statistical analyses were performed using the Minitab 13 statistical software package. (Minitab, Inc., State College, PA, USA). All results were expressed as means including their Standard Error of Means (SEM). Comparison between groups was performed using Student's t-test, and $p< 0.05$ was selected as the level required for statistical significance. Statistical comparisons between three groups were assessed by one-way analysis of variance (ANOVA). When F ratios were significant ($p<0.05$), one-way ANOVA was followed by Tukey's Post hoc test for comparisons of multiple group means.

Results

Well-known vegetables consumed commonly in Mediterranean and Aegean regions were tested for their effects on aromatase expression at protein, mRNA and activity levels and cell viability in PC-14 and PC-3 human non–small cell lung cancer cells..

Cytotoxic effect of VHE on cell viability

The cytotoxicity of VHE in PC-3 and PC-14 cells was investigated by crystal violet staining. Of these 30 different VHE, twenty VHE demonstrated significant anti-proliferative effect on PC-3 and PC-14 cells as compared to the control. The remaining ten VHE showed varying degrees of cytotoxicity on PC-3 and PC-14 cells.

Table 1 summarizes the VHE cytotoxic activity of VHE on PC-3 and PC-14 cell lines. The high level of reduction in PC-3 cell viability was observed with *Mentha piperita, Cynara scolymus, and Rhus coriaria*. Similarly, growth of PC-14 cells was inhibited by *Cynara scolymus, and Tamus communis. The IC50 values of the VHE are given in Table 2.*

Table 1: Effect of VHE on the viability of PC-3 and PC-14 cells. Confluent cell cultures were treated with 200 µg/ml of extract for 48 hours and cell viability was determined by the crystal violet staining. Viability is expressed as percent of control. Results are expressed as means of two independent experiments, with each experiment run in triplicates.

	Viability (% of Control)	
Vegetable or Herb Extract	PC-3 cells	PC-14 cells
Asparagus acutifolius	141 ± 2,83*	112 ± 6,43
Brassica oleraceae acephala	108 ± 3,54	110 ± 2,60
Spinacia oleracea	81 ± 5,65	93 ± 4,34
Laurus nobilis	32 ± 7,00*	45 ± 6,92*
Papaver somniferum	106 ± 12,1	104 ± 9,32
Sesamum indicum	127 ± 2,53*	109 ± 2,97
Petroselinum crispum	128 ± 1,13*	135 ± 0,94*
Mentha piperita	22 ± 2,67*	40 ± 2,45*
Rumex acetosa	79 ± 1,43*	101± 2,34
Lepidium sativum	43 ± 2,87*	39 ± 1,19*
Morus nigra	85 ± 1,65	94 ± 1,09
Rosmarinus officinalis	113 ± 1,28	86 ± 1,75
Anethum graveolens	93 ± 2,04	105 ± 1,29
Ocimum basilicum	87 ± 0,93	107 ± 0,87
Beta vulgaris	91 ± 0,54	105 ± 0,32
Thymus sp.	65 ± 0,45*	74 ± 1,59*
Crocus sativus	32 ± 0,91*	38 ± 0,73*
Melissa officinalis	48 ± 0,82*	64 ± 0,72*
Cynara scolymus	23 ± 1,29*	14 ± 1,28*
Papaver rhoeas	101 ± 1,83	134 ± 1,34*
Tamus communis	44 ± 0,34*	30 ± 0,29*
Emca sativa	77 ± 0,38*	87 ± 0,27
Vitex agnus	76 ± 3,98*	36 ± 3,72*
Malva sylvestris	101 ± 2,87	107 ± 1,64
Beta vulgaris var. cicla	123 ± 0,23*	117 ± 2,34*
Chenopodium sp.	81 ± 1,74	106 ± 1,93
Taraxacum sp.	109 ± 0,13	108 ± 0,95
Rhus coriaria	27 ± 1,65*	66 ± 2,93*
Allium porrum	95 ± 1,43	35 ± 2,65*
Urtica dioica	76 ± 0,34*	79 ± 1,23*

*Significantly different from respective control value ($p<0.05$)

Table 2: Effect of VHE on PC-3 and PC-14 cells viability. Cell cultures were treated with increasing concentrations of herbs for 48 hours and cell viability was determined by the crystal violet staining. Viability is expressed as a percentage of control. Results are expressed as means of two independent experiments, with each experiment run in triplicate.

	PC-3		PC-14	
Vegetable or Herb Extract	Dose (µg/ml)	Reduction in proliferation (%)	Dose (µg/ml)	Reduction in proliferation (%)
Laurus nobilis	10	55 ± 7,13*	50	53 ± 0,93*
Mentha piperita	25	56 ± 6,04*	100	50 ± 2,24*
Lepidium sativum	100	49 ± 2,24*	50	52 ± 1,39*
Crocus sativus	25	55 ± 3,31*	50	59 ± 4,61*
Cynara scolymus	50	52 ± 2,67*	10	52 ± 2,67*
Tamus communis	50	57 ± 3,06*	1	57 ± 3,06*
Vitex agnus	200	54 ± 2,31*	200	53 ± 1,20*
Rhus coriaria	50	58 ± 0,80*	50	56 ± 2,04*
Allium porrum	100	51 ± 1,84*	100	52 ± 0,20*

*Significantly different from respective control value ($p<0.05$)

Effect of VHE on aromatase activity

PC-3 and PC-14 cells were treated with VHE at IC50 concentrations, harvested after 48-h incubation, and cell lysates were used as the enzyme source. The effects of VHEs are summarized in Figure 1. VHE treatment at the IC50 concentration demonstrated that *Laurus nobilis, Mentha piperita, Crocus sativus* reduced aromatase activity 32%, 44%, 36% and 42%, 32%, 56% in PC-14 and PC-3 cell lines, respectively, when compared to the control ($p < 0.05$). The four herbs demonstrated the strongest inhibition in PC-14 cells were *Laurus nobilis, Mentha piperita, Crocus sativum* and *Allium porrum*. On the other hand, *Allium porrum* increased aromatase activity by 21% in PC-3 cell while decreasing aromatase activity 52% in PC-14 cells. MCF-7 cell was used as the positive control for aromatase activity.

In vitro inhibition of aromatase activity by selected VHEs

The four herbs, *Laurus nobilis, Mentha piperita, Crocus sativum* and *Allium porrum,* demonstrated the strongest inhibition in PC-14 cells *in vivo* were tested also for their *in vitro* inhibitions. Microsomes were prepared from untreated PC-3 and PC-14 cells and used for *in vitro* inhibition studies. Figure 2 shows the aromatase inhibition profiles of the four VHEs extract, all inhibited aromatase activity, though to a varying degree. Strikingly, *Allium porrum* decreased aromatase activity of PC-14 cells while increasing aromatase activity of PC-3 cells. MCF-7 cell was used as the positive control for aromatase activity.

Effects of VHE on aromatase protein and mRNA levels

CYP19 mRNA level was decreased by 2.5 fold but protein level was unchanged in *Allium porrum*-treated PC-14 cells. However, *CYP19* protein level was unchanged in *Allium porrum*-treated PC-3. In contrast, *CYP19* mRNA level was increased in *Allium porrum*-treated PC-3 cells (Fig. 3 and Fig. 4). Image analysis of western blots and RNA gels revealed that both the aromatase protein and RNA levels remained unchanged for *Laurus nobilis*, *Mentha piperita and Crocus sativus* treated PC-3 and PC-14 cells (Fig. 3 and Fig. 4). No significant changes were observed with other VHEs tested (Fig. 3 and Fig. 4).

Discussion

CYP19 or aromatase is responsible for the local production of estrogens, and overexpression or increased activity of this enzyme is associated with NSCLC and breast cancer [16, 17]. Given the carcinogenic properties of endogenous estrogens, reducing their levels in the body by inhibition of steroidogenic enzymes such as *CYP19* would protect against lung cancer development. With the clinical success of various synthetic aromatase inhibitors for the treatment of breast and lung cancer, researchers have been investigating the potential of natural products as aromatase inhibitors. Natural products have a long history of medicinal use in both traditional and modern societies, and have been utilized as herbal remedies, purified compounds, and as starting materials for combinatorial chemistry.

Studies have indicated that herbs contain significant amounts of bioactive phytochemicals that have antiproliferative and anticancerogenic properties. [18, 19]. Quercetin from *Allium porrum* and other related flavonoids have been shown to inhibit carcinogen-

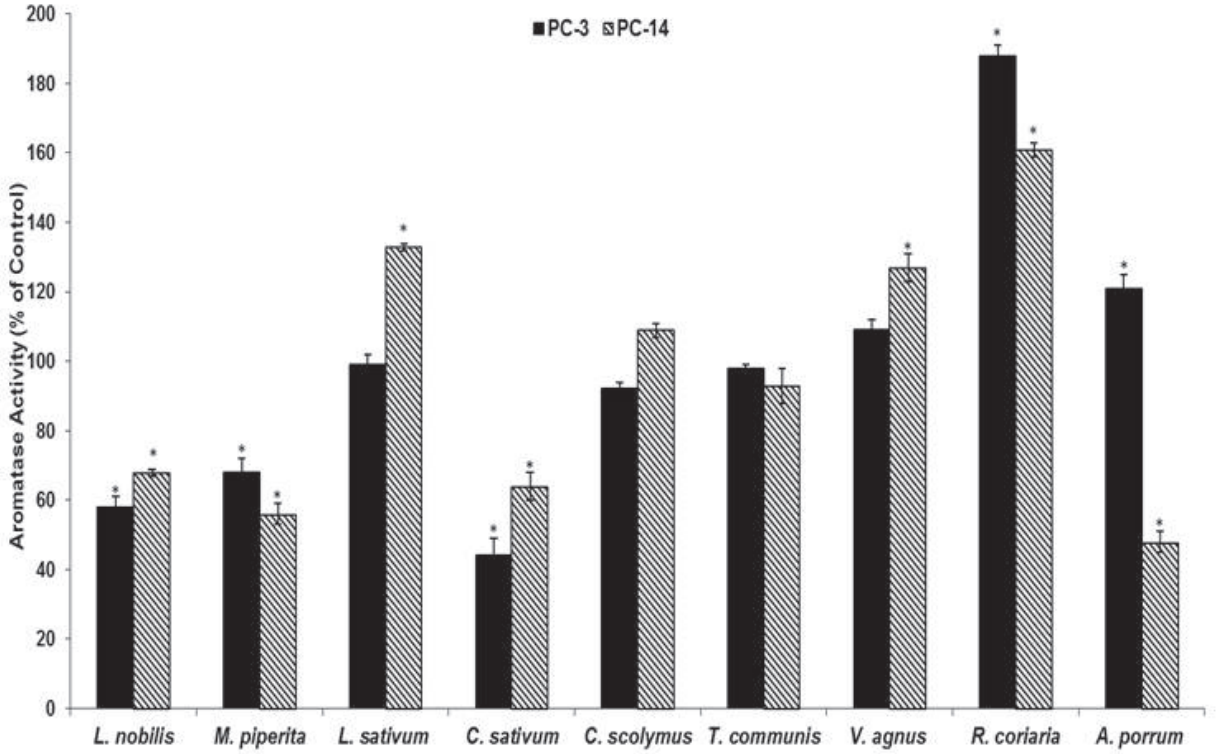

Figure 1: The effect of VHE on the *in vivo* aromatase activity of PC-3 and PC-14 cells. PC-3 and PC-14 cells were incubated with IC_{50} concentration of VHE for 48 h and aromatase activities were measured as described in Materials and Methods. Control was not treated by VHE. MCF-7 cell line was used positive control. Aromatase activity was expressed as pmol of fluorescein per mg of protein per minute. Results are expressed as means of four independent experiments, with each experiment run in triplicate. *, Significantly different from respective control value ($p<0.05$)

induced lung tumors and exert antiapoptotic effect on lung carcinoma cell lines. Furthermore, quercetin exerted inhibitory effects on aromatase and suppressed the transcription of *CYP19* mRNA in human granulosa luteal cells [19]. Similarly, the result of this study shows that *Allium porrum* reduced aromatase activity on PC-14 cell lines. In addition, although the aromatase activity and mRNA level decreased significantly, the protein level did not change. Therefore, the observed mRNA and activity decrease resulting from *Allium porrum* treatment suggest that the inhibitory effect could be transcriptional.

Allium porrum showed strong cytotoxic effect on PC-14 cell lines whereas no significant cytotoxic effect on PC-3 cell line. Although PC-3 and PC-14 cell lines are derived from lung adenocarcinoma, PC-3 cell lines express EGFR (Epidermal Growth Factor Receptor) gene mutation while PC-14 cell line has wild type EGFR. Recently, it was shown that the EGFR gene is mutated in approximately 20% of NSCLCs. And these mutations in the tyrosine kinase domain of the EGFR gene strongly correlate with substantial clinical response to EGFR inhibitors, such as gefitinib, in patients with NSCLC [20, 21]. In addition, very recent studies have shown that estrogen effects are mediated not only through nuclear estrogen receptors (ER)s but also through cytoplasmic/membrane ERs and the partners of extra-nuclear ER include PI3K and the tyrosine kinase Src [22, 23]. Furthermore, strong evidences suggest that endocrine resistance is associated with cross-talk between upstream kinases and ER [23] Therefore, EGFR might be the reason for the differential effect seen with *Allium porrum* in this study .but further studies are required.

The chemopreventive action and antimutagenic properties of *Mentha piperita* are known on lung cancer [24]. Several dietary agents including quercetin, genistein, daidzein and resveratrol have shown to be effective aromatase inhibitors in cancers [25-29]. Resveratrol from *Mentha piperita* has been shown to be effective inhibitors of several growth factor signaling pathways [30]. Genistein from *Lepidium sativum* induced apoptosis and inhibited proliferation in a variety of human cancer cell lines [31, 32]. Genistein and resveratrol improve the effectiveness of EGF receptor tyrosine kinase inhibitors in NSCLCs [33, 34]. The results of the present study show that extract obtained from *Mentha piperita* and *Laurus nobilis* reduced aromatase activity without altering protein and mRNA levels significantly in PC14

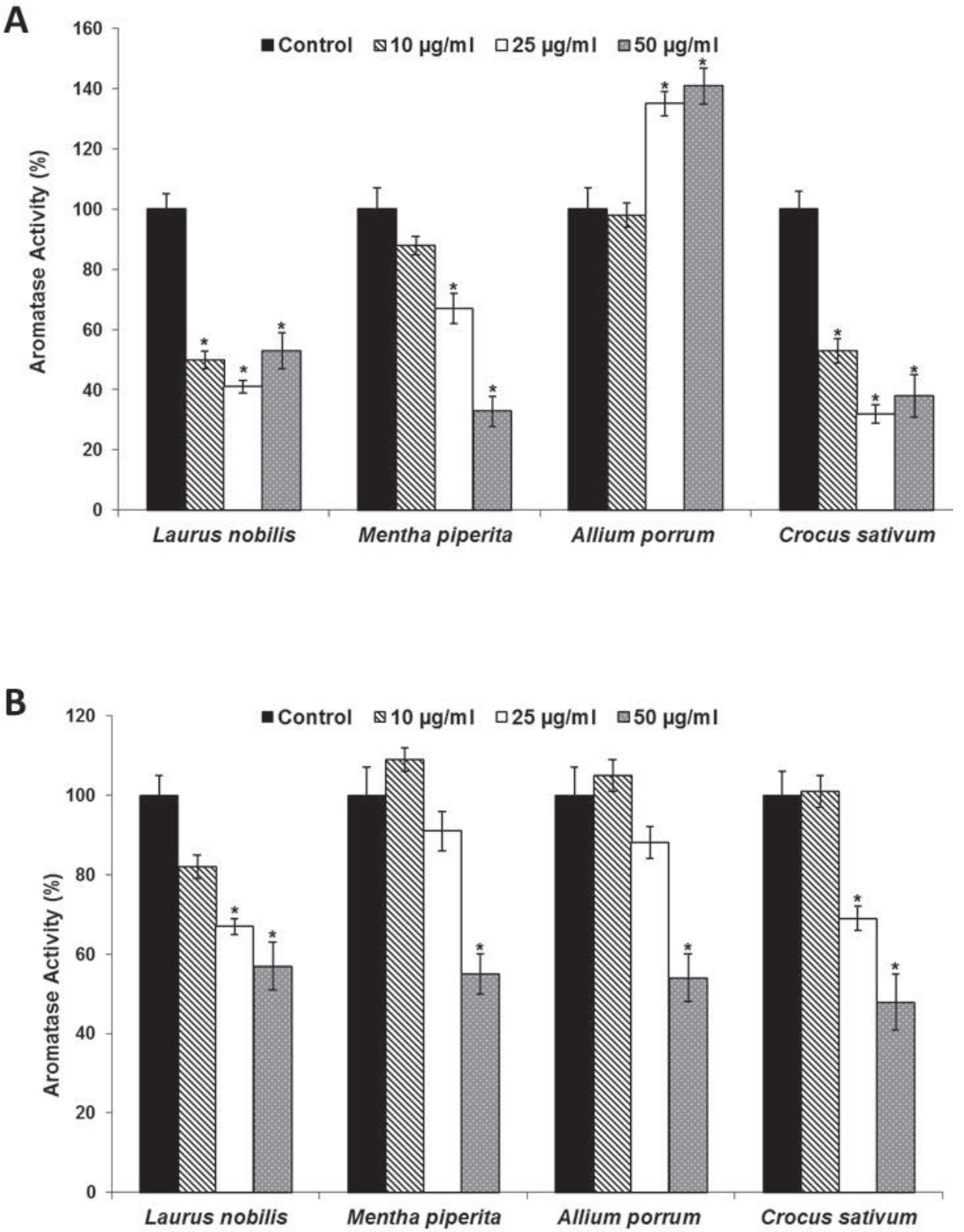

Figure 2: The effect of VHE on the *in-vivo* aromatase activity of PC-3 and PC-14 cells. (**A)** PC-3 and (**B)** PC-14 cells were collected, added varying concentration of VHE and aromatase activities were measured as described in Materials and Methods. Control cells were not treated with VHE. Results are expressed as means of four independent experiments, with each experiment run in triplicate. *, Significantly different from respective control value ($p<0.05$)

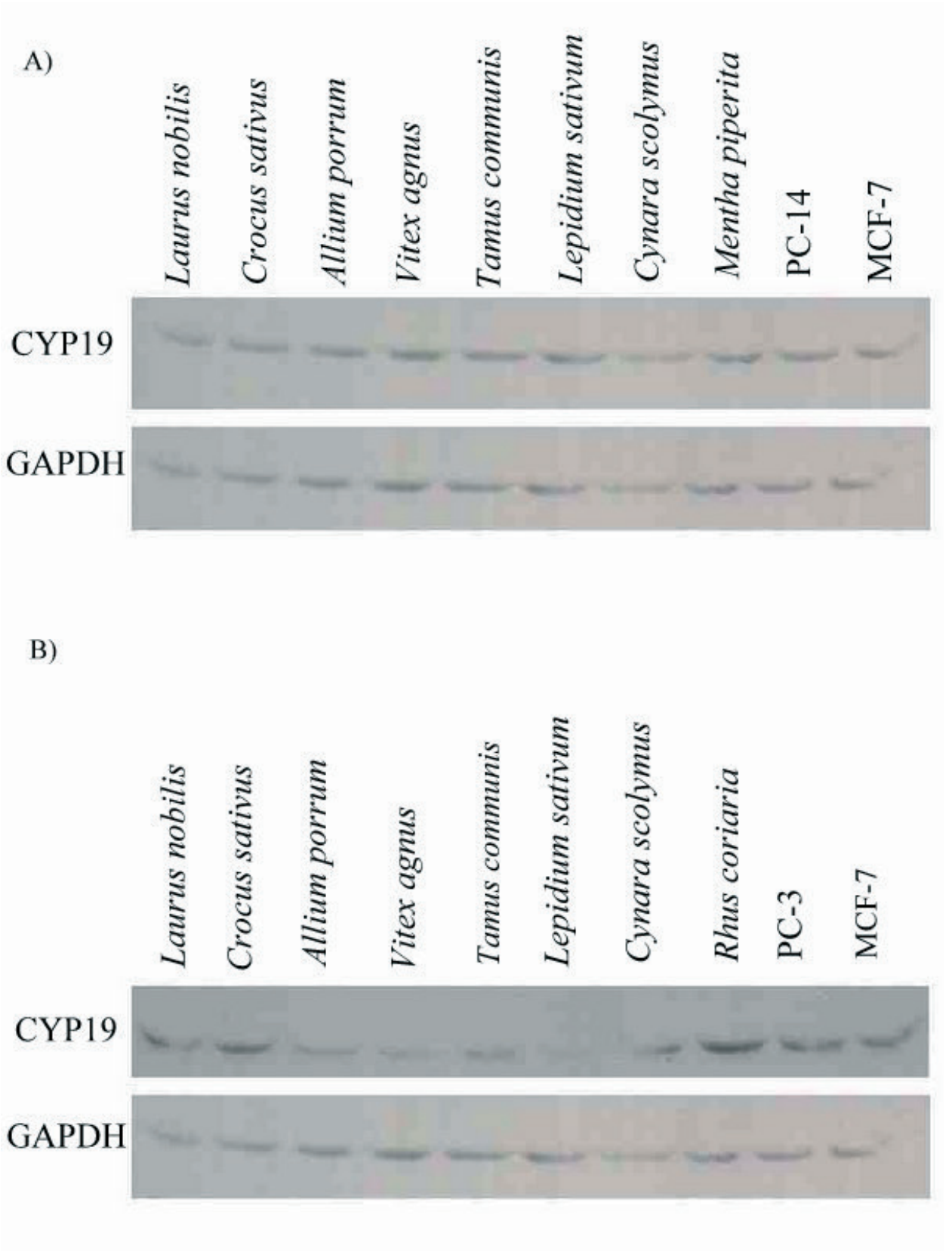

Figure 3: The expression of *CYP19* protein in control and VHE-treated cells. Treatments were carried out as described in Materials and Methods. **A.** Representative immunoblot analysis of *CYP19* and *GAPDH* proteins in PC-14 cells **B.** Representative immunoblot analysis of *CYP19* and *GAPDH* proteins in PC-3cells. MCF-7 cell was used as the positive control for aromatase activity.

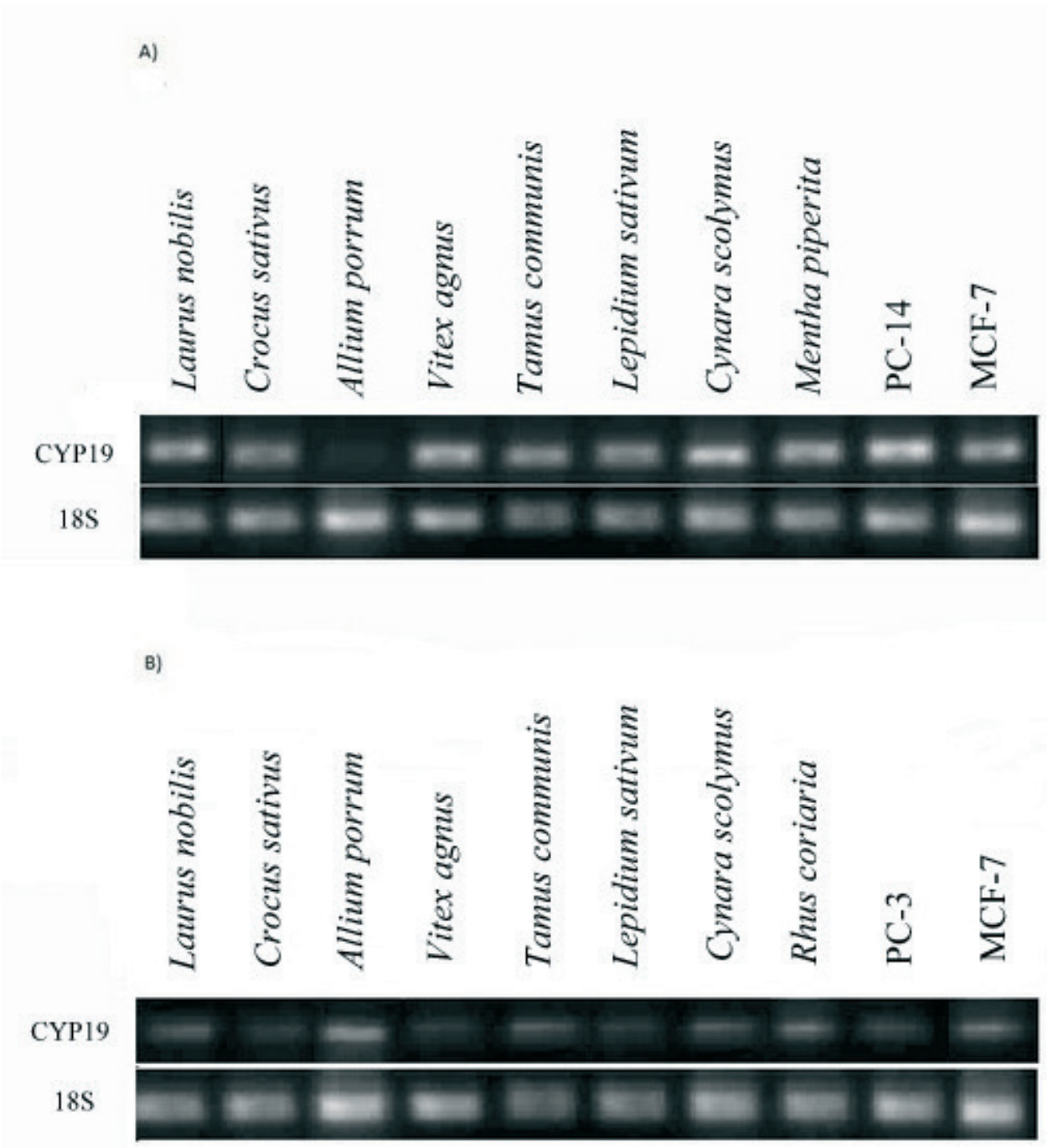

Figure 4: The expression level of CYP19 mRNA in control and VHE-treated cells. Treatments were carried out as described in Materials and Methods. Representative agarose gels showing the effect of VHE treatment on the regulation of *CYP19* mRNA expressions **(A)** in PC-14 cells and **(B)** in PC-3 cells. MCF-7 cell was used as the positive control for aromatase activity.

and/or PC3 cell lines. Therefore, the observed aromatase inhibition resulting from *Mentha piperita* and *Laurus nobilis* treatment could be neither transcriptional nor translation; this remains to be elucidated.

Very recently, saffron (*Croccus sativus*) was the candidate for its anticancer and antitumor properties and, particularly, its cytotoxic property has been studied in the breast and lung cancer cell lines [35, 36]. Similarly, present study demonstrated that *Crocus sativus* exerts cytotoxic and anticancer actions in lung cancer cell lines. As a result of these effects on lung cancer, the bioactive phytochemicals and dietary vegetables may help to protect cellular systems from cancer as well as reduce the impact of carcinogenesis.

In this study, we have investigated the effect of extracts obtained from well-known vegetable diets consumed

regularly in Mediterranean and Aegean regions of Turkey on the expression of aromatase enzyme in human non–small cell lung cancer (NSCLC) cells- PC14 and PC3 cell lines. For this purpose, we focused specifically on dietary aromatase inhibition and anti-proliferative effects of herbs on PC-3 and PC-14 cell lines. Out of the 30 vegetable and/or herbs screened, we identified four plants that exhibited dual properties on PC-3 and PC-14 cell lines. To our knowledge this is the first report on the aromatase inhibitinon capacity of the leek, bay, peppermint and saffron.

In conclusion, *Laurus nobilis* (bay), *Mentha piperita* (peppermint), *Crocus sativus* (saffron) *and Allium porrum* (leak) contain valuable aromatase inhibiting agents and consumption of these foods might be protective in development of lung cancers. Inhibitory effects of these herbs on aromatase are hitherto not known and are shown with this study. Further scientific studies should be carried out to determine the individual compounds responsible for these effects and to elucidate the molecular mechanism behind these actions.

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