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Cyclamen L. Inhibits Nitric Oxide Production in LPS-stimulated NSCLC Cells

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ABSTRACT

Cyclamen L., belonging to the Primulaceae family, is a tuberous perennial geophyte with some taxa indigenous to Turkey. However, this genus has been poorly investigated for its cytotoxic and anticancer potentials. The current study aimed to explore the antiproliferative effects of the ethanolic extracts of three *Cyclamen* taxa (*C. pseudibericum*, *C. mirabile* and *C. persicum*) and their nitric oxide (NO) inhibitory activity in LPS-stimulated non-small cell lung cancer (NSCLC) cell lines, namely HCC78 and H1975. Also, total saponin contents of the extracts were determined as quillaja equivalents. The cytotoxicity of the *Cyclamen* extracts was assessed by the CellTiter-Glo assay. *C. persicum* extract caused a higher cytotoxic effect on both H1975 and HCC78 cells than the other two *Cyclamen* extracts and its IC_{50} values in H1975 and HCC78 cells were determined to be 17.27 and 34.15 µg/ mL, respectively. While Griess reaction was performed to determine the nitrite levels as an index of NO production in LPS-stimulated NSCLC cells treated with the *Cyclamen* extracts, vanillin-sulphuric acid method was used to detect total saponin contents in the extracts. Among the three *Cyclamen* taxa evaluated, the highest inhibitory activity towards NO production in HCC78 cells was obtained with from *C. pseudibericum,* while *C. persicum* showed the highest inhibitory activity in H1975 cells. As a result, this study demonstrated that the tuber extracts of three Cyclamen taxa, which have been determined their total saponin contents, had significant cytotoxic activity and NO inhibitory potentials against HCC78 and H1975 non-small cell lung cancer cell lines. These data suggest that *Cyclamen* L. extracts examined in this study merit further research so as to isolate the bioactive secondary metabolites with anti-tumor potentials.

Keywords: *Cyclamen,* Cytotoxicity, Nitric oxide, LPS, NSCLC cell lines.

INTRODUCTION

The use of herbal products, in whole or their certain parts have been gaining considerable attention as therapeutic or prophylactic measures for many disorders and/or diseases in our daily life throughout the world. Severe adverse effects, higher cost, insufficiency, and ineffectiveness of many allopathic drugs have led the researchers to focus more on herbal medicines to combat many diseases including cancer[1].

Nitric oxide (NO), which is a short-lived gaseous free radical produced by nitric oxide synthase (NOS), has been called a "double-edged sword" with beneficial antiviral, microbicidal, immunomodulatory and antitumoral effect and deleterious effects such as inhibition of enzyme functions, alteration of deoxyribonucleic acid, induction of lipid peroxidation, mutation of tumor suppressor genes, cytotoxicity, inhibition of mitochondrial respiration, depletion of antioxidant stores and hypoxia induced angiogenesis in cancer depending on the amounts and conditions under which it is produced[2]. NO either facilitates cancer-promoting characters or act as an anti-cancer agent[3]. However, in many human cancers excessive and unregulated NO synthesis probably promote tumour

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growth and metastasis[4]. Therefore, more attention is now being paid to the development of new drugs as potent inhibitors of NO production in respect of cancer treatment[5].

Lung cancer is the leading cause of death among cancers globally. This elevated mortality is ascribed to its early metastasis, especially for non-small cell lung cancer (NSCLC)[26]. The roles of NO in lung carcinogenesis, including initiation, promotion and malignant progression, have been widely investigated[6-7]. Excessive production of endogenous and/or exogenous reactive oxygen species (ROS) and NO is implicated in the pathogenesis of lung cancer. For example, cigarette smoke, a major source of exogenous oxidants, is associated with the development of lung cancer. NO and its metabolites can lead to protein tyrosine nitration, which is elevated in lung cancer[7]. Entirely, an overpowering amount of evidence suggests a positive relationship between lung tumorigenesis and NO[8]. Although there is a large range of cytotoxic agents utilized in the cure of lung cancer, they have demonstrated problems (high toxicity, poor efficacy, different side effects etc.) in their utilization and are not as effective as anticipated[9]. Therefore, it is of great concern to find effective and better-tolerated therapeutical agents towards cancer[10]. Natural products of plant origin have drawn scientific attention for use as agents in cancer protection and treatment[11]. In this context, several plant-derived compounds such as curcumin, imperatorin and gigantol have been currently evaluated as potential anti-lung cancer agents[12].

The genus *Cyclamen* L. (Primulaceae), which is geophyte plant, is represented with 12 taxa in Turkey, 5 of which are endemic[13]. *Cyclamen* tuber extracts have been shown to possess anticancer, antimicrobial, antioxidant, analgesic and anti-inflammatory activities[11,14-16]. In a previous study, Arslan and Ozgun[17] demonstrated the cytotoxic activity of tuber extracts from *Cyclamen alpinum* (as *Cyclamen trochopteranthum*) in human cancer cell lines. Saponins are a broad class of natural compounds found in a lot of plant species, which are thought to be potential antitumor agents due to their inhibitory effects on tumor cell growth[18]. Phytochemical studies have demonstrated the presence of triterpene and triterpenoid saponins in the plants of *Cyclamen* genus[19-21]. In the light of all information mentioned above, the present study aimed to test the possible cytotoxic effect of tuber extracts from three *Cyclamen* species including *C. pseudibericum*, *C. mirabile* and *C. persicum* on NSCLC cell lines (HCC78 and H1975). Total saponin contents in the extracts were detected. The nitric oxide inhibitory activity in lipopolysaccharide (LPS)-stimulated NSCLC cell lines was also investigated in this study.

Materials and Methods

Plant material and extraction

C. pseudibericum Hildebr. (endemic), *C. mirabile* Hildebr. (endemic) and *C. persicum* Mill. were collected in 2015 from Hatay-Turkey (600 m), Mugla-Turkey (900 m) and Izmir-Turkey (15 m), respectively and identified by Professor Dr. R. Mammadov, Department of Biology, Pamukkale University, Denizli, Turkey. The tubers of plants were air-dried and reduced to a fine powder. The powdered tubers was subjected to extraction using a shaker water bath at 48-50°C for 6h with ethanol (95%) by following the method by Ozay and Mammadov[22] with slight modification. The ethanol was evaporated in a rotary evaporator and the extracts were lyophilized.

Total saponin content

Total saponin content was determined by the vanillin-sulphuric acid method. The extracts were mixed with the same amount of vanillin (8%, w/v) and twice the amount of sulphuric acid (72%, w/v). The mixture was incubated at 60° for 10 min followed by cooling in an ice water bath for 15 min. Absorbance was measured at 535 nm. The total saponin content was expressed as equivalents of Quillaja (mg QAEs/g)[23].

Cell viability assay

H1975 and HCC78 human non-small cell lung cancer cell lines (NSCLC) were used in this study. The cells were cultured in RPMI 1640 medium at 37° in a CO_2 incubator. When the cells were grown to about 90% confluence the medium was aspirated. Cells were washed, trypsinized, counted with a hemocytometer, and seeded into 96-well plates (2×10^3 cells/well). After 24 h incubation, the medium was removed from the well leaving the adherent cells and cells were treated with different concentrations of the plant extracts (1, 10, 30, 50, 75, 100, 200 µg/mL) for 72 h. For the control group, cells were not treated with any plant extract. At the end of the incubation time, cell viability was assessed by using CellTiter-Glo® mixture as recommended by supplier. ATP-based luminometric measurement from the metabolically active cells in the culture was determined by CellTiter-Glo® luminescent cell viability assay and luminescence was measured on the GloMax®-Multi Detection System (Promega). Percentage of cell viability was calculated relative to control cells.

Nitric oxide assay

The nitric oxide assay was performed as described previously with slight modification[24]. After preincubation of H1975 and HCC78 cells (2×10^3 cells/well) with LPS (1µg/mL, 24h) for NO production, the plant extracts (1, 10, 30, 50, 75, 100, 200 µg/mL) were added and incubated for 48h. Bacterial endotoxin lipopolysaccharide (LPS) causes increased inducible nitric oxide synthase (iNOS) expression and nitric oxide concentrations[25]. For the untreated control group, cells were not treated with any extracts or LPS. The quantity of nitrite in the culture medium was measured as an indicator of NO production. Amount of nitrite, a stable metabolite of NO, was determined using Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent. Afterwards, the mixture was incubated for 10 min at room temperature and the absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 560nm using a microplate reader.

Statistical analysis

Statistical analysis was performed using the software SPSS version 22.0 program. Statistical significance was determined using the one-way ANOVA. Multiple group comparisons were analyzed with Tukey's multiple comparison test. Data were expressed as a mean ± SD. *p*-value of < 0.05 was considered to be statistically significant.

Results

Total Saponin Content

The tuber extracts from *C. mirabile* and *C. persicum* in ethanol were examined for their total saponin content. Since, the total saponin content of *C. pseudibericum* was determined (160.47 ± 7.25 mg QAEs/g) in our previous study[11]. Total saponin content of *C. mirabile* and *C. persicum* was determined to be 171.52± 15.33 and 193.28 ± 21.04 mg QAEs/g, respectively.

Antiproliferative Effect of Cyclamen extracts on NSCLC Cells

The effect of three *Cyclamen* taxa on cell viability of NSCLC cells was determined by using CellTiter Glo assay. Decrease in viability in both H1975 and HCC78 cells were observed in a dose-dependent manner ($p < 0.05$) (Figure 1 and 2). According to viability assay, out of seven various concentrations (1, 10, 30, 50, 75, 100, 200 µg/mL) tested, the cytotoxic activity values (IC_{50}) of *C. mirabile, C. pseudibericum* and *C. persicum* were found as 42.98 ± 0.51, 60.52 ± 0.67 and 17.27 ± 0.37 µg/mL, respectively, for H1975 cells. As for the HCC78 cells, the calculated IC_{50} values of *C. mirabile, C. pseudibericum* and *C. persicum* were 61.82 ± 0.73, 88.61

± 0.86 and 34.15 ± 0.45 µg/mL, respectively.

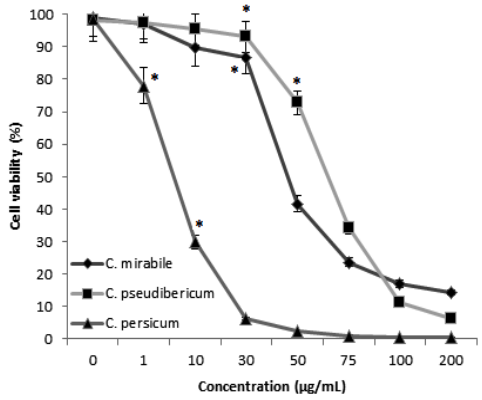

Figure 1: Cell viability of H1975 cell line treated with *C. mirabile*, *C. pseudibericum* and *C. persicum* tuber extracts. *P < 0.05 as compared with control.

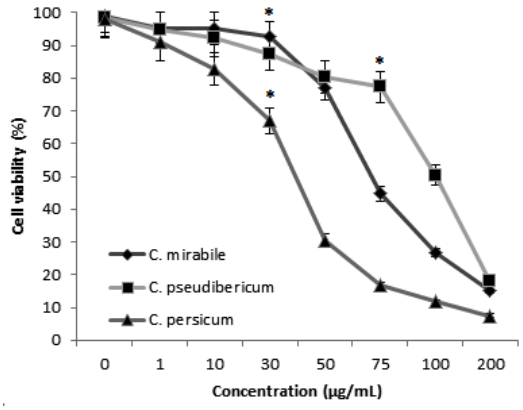

Figure 2: Cell viability of HCC78 cell line treated with *C. mirabile*, *C. pseudibericum* and *C. persicum* tuber extracts. *P < 0.05 as compared with control.

Inhibitory Effect of Cyclamen extracts on NO Production

Inhibition of NO production in LPS-activated NSCLC cells treated with the *Cyclamen* extracts was measured by using Griess reaction, as an index of NO production. We observed that the *Cyclamen* tuber extracts decreased LPS induced NO levels in non-small cell lung cancer cells. Among the three *Cyclamen* taxa evaluated, the highest inhibitory activity towards NO production in HCC78 cells was obtained from *C. pseudibericum* (26.73%), while *C. persicum* (25.08%) showed the highest inhibitory activity in H1975 cells at a concentration of 200 µg/mL ($p < 0.05$) (Figure 3 and 4). When all *Cyclamen* taxa studied are evaluated together, we found that HCC78 cell line inhibited NO production more than H1975 cell line.

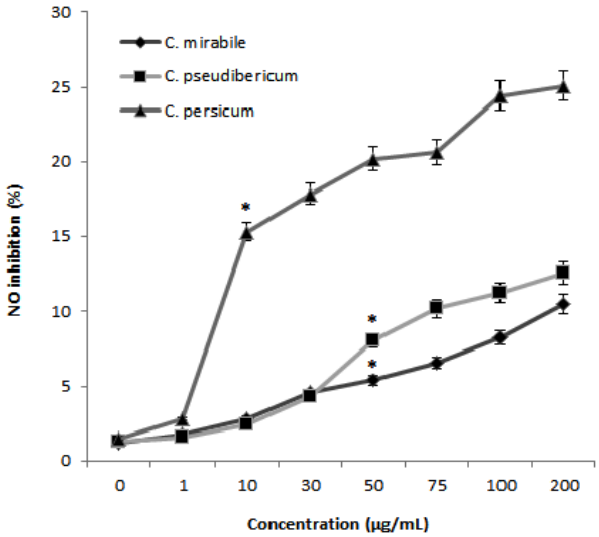

Figure 3: NO inhibition of LPS-stimulated H1975 cells by tuber extracts of *C. mirabile*, *C. pseudibericum* and *C. persicum*. *P < 0.05 as compared with control.

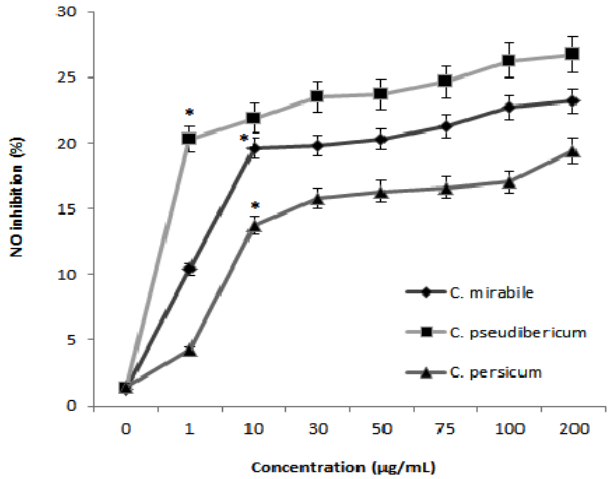

Figure 4: NO inhibition of LPS-stimulated HCC78 cells by tuber extracts of *C. mirabile*, *C. pseudibericum* and *C. persicum*. *P < 0.05 as compared with control.

Discussion

Non-small cell lung cancer is the most widespread kind of lung cancer and accounts for 85% of all lung cancers[26]. Although previous researches have shown that plant origin natural products have a big capability to reduce the risk of cancer[27-28], natural products obtained from *Cyclamen* genus have been poorly studied for cytotoxic potential. Hence, the purpose of our investigation was to detect whether *C. pseudibericum* (endemic), *C. mirabile* (endemic) and *C. persicum* tuber extracts can prevent the growth of H1975 and HCC78 NSCLC cells, and also to determine whether *Cyclamen* extracts in LPS-stimulated NSCLC cells can reduce the NO production. Total saponin contents of the extracts were also determined.

To the best of our knowledge, this researh is the first to study the effects of three *Cyclamen* taxa on proliferation and NO inhibitory activity in LPS-activated H1975 and HCC78 cells. First, we determined the total saponin contents of *Cyclamen* extracts and then assessed the cytotoxic activity of *C. pseudibericum*, *C. mirabile* and *C. persicum* tuber extracts against H1975 and HCC78 cells and found that these extracts decreased the number of cells in a concentration-dependent manner. Among the tested *Cyclamen* extracts, *C. persicum* was found to be the most cytotoxic extract with an IC_{50} value of 17.27 µg/mL on the H1975 cell line. Similarly, the most cytotoxic extract on the HCC78 cell line was found to be *C. persicum* (IC_{50} value: 34.15 µg/mL). All the tested *Cyclamen* extracts exhibited the higher cytotoxicity against H1975 cells when compared to HCC78 cells. Taking into consideration that H1975 cell line is more aggressive than HCC78, due to high metastatic capacity, it is a good result that the extracts have more cytotoxic effect on the H1975 cell line.

In a previous study, Arslan and Ozgun[17] studied the cytotoxic activity of the aqueous extract obtained from the tubers of *Cyclamen alpinum* (as *Cyclamen trochopteranthum*) in human cancer cell lines. These authors reported that the tuber extracts of *C. alpinum* had cytotoxic activity on HepG2 and Caco-2 cells, with lethal concentration (LC_{50}) values of 50 and 125 µg/mL, respectively. It is important to note that the cytotoxicity of the *C. persicum* extract used in our study was higher than that of the *C. alpinum* aqueous extract used by Arslan and Ozgun[17].

Saponin is a secondary metabolite produced by different high plant species which showed cytotoxic activity against several cancer cell lines[21]. *C. mirabile* and *C. persicum* tubers have shown to produce different saponins, such as mirabilin, cyclaminorin, cyclamin and saxifragifolin B[16,21]. In our previous study, we found that *C. pseudibericum* tuber extract exerted cytotoxic activity on A549 non-small cell lung cancer cells, with an IC_{50} value of 41.64 µg/mL. We also detected total saponin content of *C. pseudibericum* as 160.47 mg QAEs/g[11]. In the present study, we found that *C. persicum* contained more saponin than that of *C. mirabile* and *C. pseudibericum*. The higher saponin content of *C. persicum* may indicate the highest cytotoxicity.

NO is a reactive nitrogen species, which plays many roles as an effector molecule in diverse biological systems including neuronal communication, vasodilatation, antimicrobial and antitumor activities[5,29]. Although high concentrations of NO are cytotoxic, the levels produced in many human cancers possibly facilitate tumour growth and dissemination[30]. A previous study has reported that NO was found in significantly high concentration in lung cancer microenvironment[31]. Over-abundant and out-of-control NO production is related to the lung cancer pathogenesis[7]. Furthermore, clinical observation has shown that NO levels in the lungs of lung cancer patients were raised in compared to those of normal subjects[32-33]. It was reported that long-term nitric oxide exposure has been demonstrated to have major impacts on the behavior of lung cancer cells, such as enhanced cell migration[6]. Numerous studies in cell and animal models have demonstrated that NOS inhibitors

inhibit the development of cancer[34]. In this context selective inhibitors of NOS may have a curative task in specific cancers[30]. In this study, we detected that *C. pseudibericum*, *C. mirabile* and *C. persicum* species prevented NO formation in LPS-stimulated H1975 and HCC78 cells. These findings suggest significant contribution to acquire a novel bioactive compound with anticancer activity from *Cyclamen* species.

Conclusion

This study represents the first report of the impact of three *Cyclamen* (*C. pseudibericum*, *C. mirabile* and *C. persicum*) in non-small cell lung cancer cells, H1975 and HCC78. The studied tuber extracts of *Cyclamen* showed cytotoxic effect on both H1975 and HCC78 cells. In addition they inhibited NO production that can possibly raise cancer development and progression. Further studies are needed to confirm the results observed in our study and to explore the phytochemical composition of the extracts, especially saponins, which thought to be responsible for these effects.

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يمنع .Cyclamen L إنتاج أكسيد النيتريك في خلايا NSCLC المحفزة LPS

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ملخص

.Cyclamen L، ينتمي إلى عائلة Primulaceae، هو جيوفي معمر درني مع بعض الأصناف الأصلية لتركيا. ومع ذلك، تم التحقيق في هذا الجنس بشكل سيئ لإمكاناته السامة للخلايا ومضادات السرطان. تهدف الدراسة الحالية إلى استكشاف التأثيرات المضادة للتكاثر لمستخلصات الإيثانول لثلاث أصناف سيكلامين (C. pseudibericum و C. mirabile و C. persicum) ونشاط مثبط لأكسيد النيتريك (NO) في سرطان الرئة غير المحفز بالخلايا الصغيرة) خطوط الخلايا NSCLC، وهي HCC78 و H1975. أيضا، تم تحديد محتويات السابونين الكلية من المستخلصات كمكافئات .quillaja تم تقييم السمية الخلوية لمستخلصات بخور مريم بخلايا .CellTiter-Glo تسبب مستخلص C. persicum في حدوث تأثير سام أعلى للخلايا على كل من خلايا H1975 و HCC78 مقارنة بمستخلصي Cyclamen الآخرين وقيم IC50 في خلايا H1975 و HCC78 لتكون 17.27 و 34.15 ميكروغرام / مل، على التوالي.بينما تم إجراء تفاعل Griess لتحديد مستويات النتريت كمؤشر للإنتاج NO في خلايا NSCLC المحفزة LPS التي تم معالجتها بمستخلصات Cyclamen، تم استخدام طريقة حمض الفانيلين-الكبريتيك للكشف عن محتويات saponin الكلية في المستخلصات. من بين الأصناف الثلاثة من Cyclamen التي تم تقييمها، تم الحصول على أعلى نشاط مثبط تجاه إنتاج NO في خلايا HCC78 باستخدام C. pseudibericum، بينما أظهر C. persicum أعلى نشاط مثبط في خلايا .H1975 ونتيجة لذلك، أظهرت هذه الدراسة أن مستخلصات الدرنة لثلاث أصناف سيكلامين، والتي تم تحديد محتوياتها الكلية من السابونين، كان لها نشاط سام كبير للخلايا ولا توجد إمكانات مثبطة ضد HCC78 و H1975 خطوط خلايا سرطان الرئة غير الصغيرة. تشير هذه البيانات إلى أن مقتطفات .Cyclamen L التي تم فحصها في هذه الدراسة تستحق مزيدًا من البحث وذلك لعزل المستقلبات النشطة بيولوجيًا ذات الإمكانات المضادة للورم.

الكلمات الدالة: خطوط الخلايا NSCLC، LPS، سمية خلوية، أكسيد النيتريك، بخور مريم.

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