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Original Article

The Role of *Cydonia oblonga, Portulaca oleracea,* and *Artemisia dracunculus* on Hypoxia

Mohammad Hossein Hosseinzadeh[1#], Mohammad Eghbali[1#], Zahra Hashemi[2], Narges Naserirad[3], Mahbube Shirdel[3], Melika Rafizadeh[3], Davood Farzin[1], Mohammad Ali Ebrahimzadeh[1*]

[1]Pharmaceutical Sciences Research Center, Hemoglobinopathy Institute, Mazandaran University of Medical Sciences, Sari, Iran
[2]Department of Medicinal Chemistry, Faculty of Pharmacy, Ayatollah Amoli Branch, Islamic Azad University, Amol, Iran
[3]Ramsar International Campus, Pardis School of Pharmacy, Mazandaran University of Medical Sciences, Ramsar, Iran

Abstract

Background: Hypoxia exists in some malignancies and is a prognostic risk factor contributing to tumor growth and metastasis. Anti-hypoxic compounds may improve this situation and be considered as anti-cancer agents. In previous reports, *Cydonia oblonga*, *Portulaca oleracea*, and *Artemisia dracunculus* showed anti-cancer activities. So, we investigated the anti-hypoxic activities of *C. oblonga*, *P. oleracea*, and *A. dracunculus* to evaluate the possible mechanism of their effectiveness in treating cancer.

Methods: Total phenolic and flavonoid contents and HPLC analysis were performed on *C. oblonga* leaves, *P. oleracea,* and *A. dracunculus* aerial parts extract. Anti-hypoxic activities were evaluated in asphyctic, haemic, and circulatory hypoxia models.

Results: *A. dracunculus* extract (at 250 mg/kg) significantly improved the survival time compared to the normal saline ($P<0.0001$) in asphyctic hypoxia, even its effect was significantly better than phenytoin in this dose ($P=0.0005$). Although the extracts increased the survival time in other doses, their effects were not significant ($P>0.05$). In haemic hypoxia, the extracts were ineffective at any dose ($P>0.05$). At 250 mg/kg, *P. oleracea* and *A. dracunculus* significantly increased the survival time ($P<0.001$ and $P<0.05$, respectively) in circulatory hypoxia. Their effects were similar to propranolol ($P>0.05$).

Conclusion: The anti-cancer effects of *C. oblonga* are not dependent on the anti-hypoxic effects. *P. oleracea* and *A. dracunculus* have anti-hypoxic effects only in high doses, indicating their extracts' weak anti-hypoxic ability or the presence of potent anti-hypoxic compounds with low concentrations in them.

Keywords: Neoplasms, Hypoxia, Quince, Purslane, Tarragon

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Introduction

Free radicals are a group of radicals with high reactivity and react with other molecules. One of the most important free radical species is reactive oxygen species (ROS). The oxidation and production of ROS are a necessary part of our lives involved in critical biological reactions. However, overproduction of ROS can cause severe damage to cells and tissues due to the oxidation of lipids, proteins, and DNA (1,2). Hypoxia is lack of oxygen in the body that can occur in physiological conditions due to pulmonary ventilation reduction, climbing, and heavy physical exercise or in pathological conditions such as bleeding, ischemia, poisoning, stroke, and some cardiovascular diseases. When the cell senses a lack of oxygen, it releases ROS from the mitochondria as a signalling agent. ROS release aims to activate a cascade of reactions that provide the conditions for confrontation with hypoxia in the medium and long term. However, in acute and severe hypoxia, excessive release of ROS produces mediators that lead to cell death and tissue necrosis. Therefore, failure to treat hypoxia can cause severe injury and death (2,3). A recent study showed that an effective compound in treating hypoxia reduces cell ROS, lipid peroxidation, and protein oxidation (4). Also, compounds with good antioxidant effects have been reported to have significant anti-hypoxic effects (5,6). Therefore, antioxidants probably have anti-hypoxic effects.

The amount of oxygen available in malignant and tumor tissues is insufficient. Normal cells may die due to the decreased oxygen supply. However, many malignant and tumor cells can survive in this hypoxic environment (7). Chemotherapy and radiation are ineffective against tumor cells in this condition. Hypoxia exists in some malignancies, such as head and neck carcinoma, and is

*Corresponding Author: Mohammad Ali Ebrahimzadeh, Email: zadeh20@gmail.com
#Both authors contributed equally to this paper.

a prognostic risk factor contributing to tumor growth and metastasis through various pathways. So, anti-hypoxic compounds may improve this situation and be considered as anticancer agents in the future (8).

Cydonia oblonga is a plant from the Rosaceae family, with the common name quince (called Beh in Persian medicine). In traditional medicine, bronchitis, cough, fever, nausea, diarrhea, constipation, cystitis, hemorrhoids, diabetes, and hypertension have been treated with different parts of quince (9). Quince fruits are also consumed as marmalade or jelly (10). It has antioxidant and anti-carcinogenic properties (9). Quince has an antiproliferative effect on the colon (Caco-2 cells) and renal cancer (human renal epithelial cancer cells) (11). Also, the cytotoxic effects of quince were assessed on cervical carcinoma (HeLa), lung epithelial (A549), and human hepatoblastoma (HepG2) cell lines (12).

Portulaca oleracea is an important medicinal plant with the common name of purslane from the Portulacaceae family (13). Antioxidant and anti-carcinogenic activities are some of the pharmacological effects of purslane (14). The total phenolic and flavonoid content of the methanol extract of purslane was highly associated with its antioxidant activity (15). Also, polysaccharide components (16,17), sulfated derivatives (18,19), some alkaloids (20), isoflavonoids (21), and seed extract of purslane showed an antiproliferative effect on human hepatoma (HepG2), uterine cervix carcinoma (Hela), human lung cancer (A549), and human gastric (SGC-7901) cell lines.

Tarragon is the common name for *Artemisia dracunculus* (AD), which belongs to the Asteraceae (Compositae) family. Because of the presence of monoterpenes and sesquiterpenes, this plant has a distinct odor, which is why it is used in traditional medicine and widely cultivated in Asia, Europe, and America (22). Tarragon is valuable for its antioxidant, anticancer, and immunomodulatory properties (23). Tarragon leaves are effective in treating lymphoma (L5178YD) and esophageal squamous carcinoma (Eca-109) cell lines (24,25).

Therefore, considering the antioxidant and anticancer effects of *C. oblonga*, *P. oleracea*, and *A. dracunculus* in previous studies, we measured their anti-hypoxic activities to evaluate a possible mechanism of their effectiveness in treating neoplasms.

Material and Methods

Collection of plant and preparation of the extract

The *C. oblonga* leaves, *P. oleracea,* and *A. dracunculus* aerial parts were collected, authenticated, dried at room temperature, and powdered. Extraction was performed based on the maceration method with methanol. Briefly, 10 g of each powder was exposed to 100 mL of methanol for 72 hours at room temperature separately (the procedure was repeated three times). The extracts were collected, filtered with filter paper, and concentrated with a rotary evaporator (6). The yield was 18.26%, 11.05%, and 13.94% for *C. oblonga*, *P. oleracea*, and *A. dracunculus,* respectively.

Determination of total phenolic content

The Total phenolic compounds were measured by Folin-Ciocalteu method. Different concentrations of gallic acid (100, 50, 25, 12.5, 6.25, and 3.75 µg/mL) were used as standard and 0.5 mL of each concentration of gallic acid was mixed separately with 2.5 mL Folin reagent (0.1 N) and 2 mL sodium carbonate (75 g/L). After 2 hours, the adsorptions of the solutions were measured using a UV/VIS spectrophotometer at 725 nm against the blank, and the standard curve was drawn. The same process was performed for 0.5 mL of extracts (500 µg/mL), and the total phenolic content was reported based on mg gallic acid equivalent/g dried extract (26).

Determination of total flavonoid content

Different concentrations of quercetin (250, 125, 62.5, 31.25, and 15.62 µg/mL) were used as standard and 0.5 ml of each concentration was mixed with 1.5 mL methanol, 0.1 mL aluminum chloride (10%), 0.1 mL potassium acetate (1 M), and 2.8 mL distilled water and left at room temperature for 30 minutes. Finally, the absorbance was measured against the blank at 415 nm, and the standard curve was drawn. The same process was performed for 0.5 ml of extracts (500 µg/mL), and the total flavonoid content was reported based on the mg quercetin equivalent/g dried extract (27).

High-pressure liquid chromatography

The phenolic compounds of *C. oblonga*, *P. oleracea*, and *A. dracunculus* extracts were analyzed by the HPLC method. The HPLC system consisted of a model K-1001 solvent delivery system equipped with a Rheodyne injection valve (20 mL sample loop inserted) and a UV-Vis spectrophotometric detector model, K-2600 set at 290 (for gallic acid) and 320 nm (for caffeic acid, ferulic acid, and rutin) (all from Knauer Assoc., Germany). The analysis was performed using an ODS-C18 column (250 × 4.6 mm I.D., 5 mm particle size, Shim-pack VP-ODS).

All solvents were filtered and degassed before entering the column. The mobile phase was acetonitrile: water: formic acid (16: 83.8: 0.2) aqueous solution at pH 2.5. was used for separation. All separations were performed at 25 °C with a 1 mL/min flow rate and a 20 minutes run time. The mentioned assay was applied to determine the extract by retention times and UV spectral peaks of the sample with authentic standards (Figure 1; standards).

Animals

Male Swiss albino mice (30.17 ± 4.92 g) were divided into groups of six and were housed at 25.3 ± 3.2 °C and 45%-

55% relative humidity, 12 hours light: 12 hours dark period (light at 7 AM) in polypropylene cages. The animals had free access to standard pellets and water The tests took place between 8:00 and 15:00 hours. All the laboratory experiments were carried out on the Laboratory Animal Care and Use protocols of the NIH. The experimental procedure was also accepted by the Institutional Animal Ethics Committee of the Mazandaran University of Medical Sciences (ethics numbers: IR.MAZUMS.RIB.REC.1400.042 for *C. oblonga* and IR.MAZUMS.RIB.REC.1400.043 for *P. oleracea* and *A. dracunculus*).

Asphyctic hypoxia

In intervention groups, mice received single i.p. injections of different concentrations of extracts (the initial dose was selected based on previous studies at 125 mg/kg and then increased or decreased based on the observed response). After 30 minutes, the animals experienced hypoxia by placing separately in a tightly closed 300 mL glass container. The animals had convulsions and 1.5-2 minutes later, they died of hypoxia. Death latencies have been recorded. The same process was done in control groups. Phenytoin (50 mg/kg) was used as the positive control, and normal saline was used as the negative control (5,6,28).

Haemic hypoxia

The haemic hypoxia test was performed based on previous reports (5,6). In intervention groups, mice received single i.p. injections of different concentrations of extracts (the initial dose was selected based on previous studies at 125 mg/kg and then increased or decreased based on the observed response). After 30 minutes, 360 mg/kg sodium nitrite ($NaNO_2$) was injected intraperitoneally and death latencies were recorded. The same process was done in control groups with the difference that the negative control was treated with normal saline (10 mL/kg), and propranolol (20 mg/kg) was used as the positive control.

Circulatory hypoxia

The circulatory hypoxia test was performed based on previous reports and with some modifications (5,6). In intervention groups, mice received single i.p. injections of different concentrations of extracts (the initial dose was selected based on previous studies at 125 mg/kg and then increased or decreased based on the observed response). After 30 minutes, 150 mg/kg sodium fluoride (NaF) was injected intraperitoneally and death latencies were recorded. The same process was done in control groups with the difference that the negative control was treated with normal saline (10 mL/kg), and propranolol (20 and 30 mg/kg) was used as the positive control.

Statistical analysis

For Statistical Analysis, GraphPad Prism 8 was used. Data were given as mean ± SD. One-way analysis of variance (ANOVA) was done. Tukey multiple comparisons test was used to determine the differences in means. All of the *P* values below 0.05 were considered significant.

Results

Total phenolic content and total flavonoid content of the extract

Total phenolic contents of *C. oblonga*, *P. oleracea*, and *A. dracunculus* were 260.63 ± 10.02, 107.42 ± 0.57, and 139.67 ± 4.47 mg gallic acid equivalent/g of dried extract ($y = 0.0048x + 0.0775$, $R^2 = 0.9953$) and total flavonoid contents were 66.65 ± 0.48, 73.10 ± 2.13, and 63.96 ± 2.59 mg quercetin equivalent/g of dried extract ($y = 0.0064x - 0.0076$, $R^2 = 0.9998$), respectively.

High-pressure liquid chromatography

The established HPLC method using a C18 column (250 × 4.6 mm, 5 mm) and acetonitrile: water: formic acid (20:79.8:0.2) as a mobile phase, flow rate of 1 mL/min could separate three phenolic acids and one flavonoid within 20 minutes (Figure 1). *C. oblonga* extract contained two phenolic and flavonoid compounds tested, including gallic acid (7.8 mg/g of extract), caffeic acid (3.9 mg/g of extract) and rutin (1.1 mg/g of extract). *P. oleracea* extract contained mainly ferulic acid (0.8 mg/g of extract) and lower amounts of gallic acid (0.6 mg/g of extract) and caffeic acid (0.5 mg/g of extract). *A. dracunculus* extract contained two phenolic acids and one flavonoid compound tested, including gallic acid (1.7 mg/g of extract), caffeic acid (3.2 mg/g of extract) and rutin (1.4 mg/g of extract).

Anti-hypoxic activity of the extract

Anti-hypoxic activities of *C. oblonga* leaves, *P. oleracea* aerial parts, and *A. dracunculus* aerial parts in asphyctic, haemic, and circulatory hypoxia have been reported in Table 1.

In asphyctic hypoxia, *A. dracunculus* extract at a dose of 250 mg/kg significantly improved the survival time compared to the normal saline ($P < 0.0001$), even its effect was significantly better than phenytoin in this dose ($P = 0.0005$). Although the extracts increased the survival time in other doses (except *P. oleracea* at 125 mg/kg), their effects were not significant ($P > 0.05$). In haemic hypoxia, the extracts were ineffective at any dose ($P > 0.05$). At 250 mg/kg, *P. oleracea* and *A. dracunculus* significantly increased survival time ($P < 0.001$ and $P < 0.05$, respectively) in circulatory hypoxia. Their effects were similar to propranolol ($P > 0.9999$ and $P = 0.4036$, respectively).

Discussion

As mentioned, an increase in cell number in cancerous tissues leads to hypoxia and overproduction of ROS.

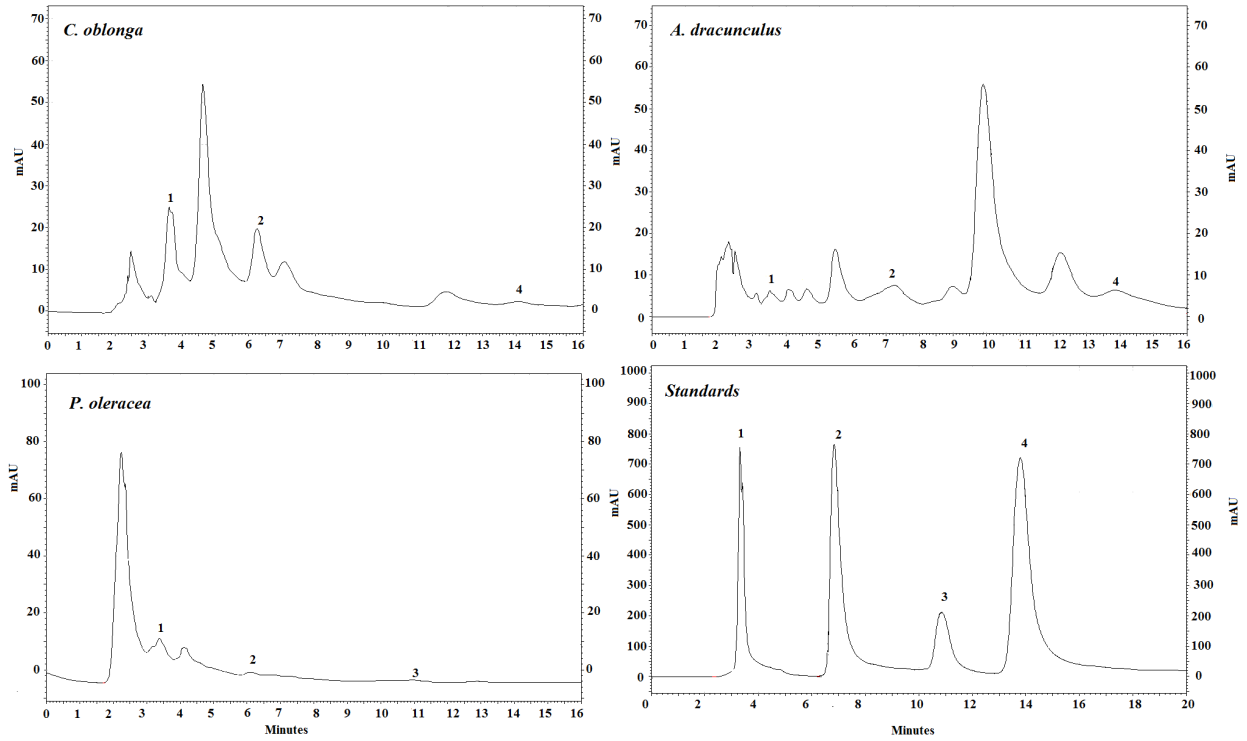

Figure 1. HPLC profiles of *C. oblonga*, *P. oleracea*, and *A. dracunculus* extracts analyzed at 280 nm. 1: gallic acid; 2: caffeic acid; 3: ferulic acid; 4: rutin (mg/g of extract)

Table 1. Anti-hypoxic activities of *C. oblonga*, *P. oleracea*, and *A. dracunculus* extracts in asphyctic, haemic, and circulatory hypoxia

Groups	Dose (mg/kg)	Asphyctic hypoxia (min)	Haemic hypoxia (min)	Circulatory hypoxia (min)
Control	-	19.20±2.24	10.50±0.66	10.11±0.86
C. oblonga	125	21.00±3.82 ^{ns}	10.25±0.59 ^{ns}	12.23±1.39 ^{ns}
	250	22.68±1.04 ^{ns}	10.31±0.49 ^{ns}	10.22±1.03 ^{ns}
P. oleracea	125	18.49±1.09 ^{ns}	11.56±1.48 ^{ns}	11.01±1.69 ^{ns}
	250	20.86±2.42 ^{ns}	10.75±1.47 ^{ns}	16.33±2.56 ***
A. dracunculus	125	22.46±2.78 ^{ns}	11.81±0.98 ^{ns}	11.20±0.78 ^{ns}
	250	40.55±6.13 ****	9.38±0.19 ^{ns}	13.57±2.06 *
Phenytoin	50	30.10±2.25 ***	-	-
Propranolol	20	-	16.39±1.83 ****	-
	30	-	-	15.91±1.59 ***

Data are expressed as mean±SD (n=6), (ns: not significant, $P>0.05$, * $P<0.05$, *** $P<0.001$, **** $P<0.0001$, compared to the negative control)

These factors can lead to the death of normal cells in the body and eventually metastasis of cancer cells. Antioxidants can effectively reduce cell death and slow cancer progression by inhibiting ROS. On the other hand, anti-hypoxic compounds may increase the chances of normal cells survival by increasing the ability of these cells to inhibit hypoxia and be effective in improving cancer. Therefore, in this study, we evaluated the anti-hypoxic activity of *C. oblonga*, *P. oleracea*, and *A. dracunculus* which were effective in treating cancer to suggest another possible mechanism for the effectiveness of these plants in the treatment of cancer.

Decreased oxygen availability in asphyctic hypoxia, converting hemoglobin to methemoglobin by $NaNO_2$ in haemic hypoxia, and lysis of hemoglobin by NaF in circulatory hypoxia reduces cellular oxygen availability and cause hypoxia in tissues (5). Compounds that can increase cell resistance in these conditions can increase the survival time and are considered anti-hypoxic compounds.

Cydonia oblonga leaf has higher phenolic content and antioxidant activity compared to peel, pulp, and seed (EC50 of 21.6, 600, 1700, and 2000 µg/mL, respectively) (29). Also, it has a potent reducing power activity (30). Methanolic extracts of *C. oblonga* leaves inhibit the development of human colon cancer (Caco-2) cells in a

concentration-dependent manner (11). *C. oblonga* extract ingredients can interact with erythrocyte membrane lipids that stop lipid oxidation by free radicals in redox reactions and prevent their diffusion by reducing the mobility of the membrane's hydrophilic and hydrophobic regions (31). It might be helpful in circulatory hypoxia. Therefore, we used *C. oblonga* leaves to evaluate the anti-hypoxic activities. Despite its good antioxidant, anti-cancer, and membrane stabilization properties, methanolic extract of *C. oblonga* leaves did not significantly affect any tests.

Portulaca oleracea reduces malondialdehyde and ROS formation and increases *superoxide dismutase* and glutathione (32), especially in blood cells (33). Its antioxidant properties are related to gallotannins, ascorbic acid, kaempferol, α-tocopherols, apigenin, and quercetin (34). Isoflavonoids from aerial parts of *P. oleracea* demonstrated significant anticancer effects against human gastric cancer cell line (SGC-7901) (35). Also, *P. oleracea* can cause considerable cytotoxicity and growth suppression in human lung cancer (A-549) cell lines (36). Genistein is one of the *P. oleracea* flavonoids (37). Treatment of A549 cells with genistein reduced proliferation and enhanced the apoptotic rate of A549 cells in a dose-dependent manner (38). So, *P. oleracea* may be effective in treating cancer by inhibiting hypoxia. Chen et al evaluated the anti-hypoxic activity of *P. oleracea* in oral administration (100, 200, and 400 mg/kg) in the hypoxia model of mice. It increased glycolysis enzyme activity, ATP levels, and survival time in a dose-dependent manner. *P. oleracea* effects were mediated through sedation or motor impairments (39). In the present study, *P. oleracea* (250 mg/kg) showed similar effects to propranolol (30 mg/kg) in circulatory hypoxia, which can confirm the Chen et al study results.

Artemisia dracunculus has considerable antioxidant activity (40). *A. dracunculus* extract inhibits lipid peroxidation by decreasing malondialdehyde and sialic acid accumulation. Also, *A. dracunculus* essential oils had radical scavenging activity (41). Hydromethanolic extracts of *A. dracunculus* (collected from various locations in Iran) have remarkable ferric reducing antioxidant power associated with total phenolic and flavonoid contents (42). Some researchers have evaluated the anticancer activity of *A. dracunculus*. It reduced the detrimental consequences of tumors in cancerous rats (43). Navarro-Salcedo et al investigated the effect of *A. dracunculus* leaf extract on mouse lymphoma cell proliferation. The anti-tumor activity of the acetonitrile extract is correlated with the concentration of polyphenols (24). Ethanolic extract of *A. dracunculus* significantly increased the survival rate in rats with acute hypobaric anoxia, while its aqueous extract had no effect (41). In this study, *A. dracunculus* methanolic extract significantly increased survival time in asphyctic and circulatory hypoxia at the highest dose (250 mg/kg).

Recently, significant effects have been reported for the ethanolic extract of *Aloysia citrodora* leaves in asphyctic hypoxia and circulatory hypoxia models. *A. citrodora* (250 mg/kg) increased the survival time significantly compared to the control group in asphyctic hypoxia ($P<0.001$), which was not significantly different from the phenytoin ($P>0.05$). The anti-hypoxic activity of *A. citrodora* (250 mg/kg) was not only much more significant than the control group ($P<0.0001$) but also stronger than propranolol in circulatory hypoxia (6). Also, significant activities have been reported for methanolic extract *of Hibiscus rosa-sinensis* in asphyctic, haemic, and circulatory hypoxia (5). *A. dracunculus* increased the survival time in asphyctic hypoxia even more than *A. citrodora* and *H. rosa-sinensis*. *P. oleracea* showed similar activity in circulatory hypoxia compared to *A. citrodora* and *H. rosa-sinensis*. *C. oblonga* could not significantly affect any of the tests. However, the methanolic extract of *Ginkgo biloba* showed weak anti-hypoxic effects, too (44).

Although controlling hypoxia is important in improving cancer, the effects of hypoxia on cardiovascular disease and COVID-19 have been studied (45,46). Even dexamethasone (used to treat COVID-19) has been reported to have anti-hypoxic effects (47). It seems that further research on the applications of anti-hypoxic compounds may lead to the discovery of vital compounds in treating various diseases.

Conclusion

It seems that anti-hypoxic compounds can be effective in treating cancer. Even some anti-cancer compounds may be effective in treating cancer by inhibiting hypoxia. Although previous studies have reported good anti-cancer effects of *C. oblonga*, *P. oleracea*, and *A. dracunculus*, according to the results, the anti-cancer effects of *C. oblonga* are not dependent on the anti-hypoxic effects. Also, *P. oleracea* and *A. dracunculus* have anti-hypoxic effects only in high doses, indicating their extracts' weak anti-hypoxic ability or strong anti-hypoxic compounds with low concentrations in them. Therefore, more studies are needed to evaluate the characteristics of *P. oleracea*, and *A. dracunculus*.

Authors' Contribution

Conceptualization: Mohammad Ali Ebrahimzadeh, Davood Farzin.
Data curation: Mohammad Hossein Hosseinzadeh, Mohammad Ali Ebrahimzadeh.
Formal Analysis: Mohammad Ali Ebrahimzadeh, Mohammad Hossein Hosseinzadeh.
Funding acquisition: Mohammad Ali Ebrahimzadeh.
Investigation: Mohammad Hossein Hosseinzadeh, Mohammad Eghbali, Zahra Hashemi, Narges Naserirad, Mahbube Shirdel, Melika Rafizadeh, Davood Farzin, Mohammad Ali Ebrahimzadeh.
Methodology: Mohammad Hossein Hosseinzadeh, Mohammad Eghbali, Zahra Hashemi, Narges Naserirad, Mahbube Shirdel, Melika Rafizadeh, Davood Farzin, Mohammad Ali Ebrahimzadeh.
Project administration: Mohammad Hossein Hosseinzadeh, Mohammad Eghbali, Mohammad Ali Ebrahimzadeh.

Resources: Mohammad Ali Ebrahimzadeh.
Software: Mohammad Ali Ebrahimzadeh, Mohammad Hossein Hosseinzadeh.
Supervision: Mohammad Ali Ebrahimzadeh.
Validation: Mohammad Ali Ebrahimzadeh, Mohammad Hossein Hosseinzadeh.
Visualization: Mohammad Hossein Hosseinzadeh, Mohammad Eghbali, Zahra Hashemi, Mohammad Ali Ebrahimzadeh.
Writing–original draft: Mohammad Hossein Hosseinzadeh, Mohammad Eghbali, Zahra Hashemi.
Writing–review & editing: Davood Farzin, Mohammad Ali Ebrahimzadeh.

Competing Interests
The authors declare no conflict of interest.

Ethical Approval
This study was approved by the Institutional Animal Ethics Committee of the Mazandaran University of Medical Sciences (ethics numbers: IR.MAZUMS.RIB.REC.1400.042 for *C. oblonga* and IR.MAZUMS.RIB.REC.1400.043 for *P. oleracea* and *A. dracunculus*).

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