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Protective effects of methanolic extract of *Vicia cracca* against hypoxia-induced lethality in mice

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ABSTRACT

Vicia genus has 45 species in Iran. Many protective and biological activities have been reported from these species. In spite of many works, nothing is known about protective effect of *V. cracca* against hypoxia conditions. In this study, protective effects of *V. cracca* extract against hypoxia-induced lethality in mice were evaluated by three experimental models of hypoxia, asphyctic, haemic and circulatory. Statistically significant protective activities were observed in some doses of extract in three models. Antihypoxic activity was especially pronounced in asphyctic model. Extract at 200 mg/kg prolonged survival time (27.37 ± 4.0 min) but was not comparable with that of phenytoin (39.80 ± 1.92). At 100 mg/kg it also prolonged survival time (24.76 ± 3.7 min) which was so higher than control group. In haemic model, *V. cracca* extract significantly and dose dependently prolonged survival time as compared to control group. At 200 mg/kg, extract was being capable of keeping the mice alive for 15.38 ± 1.93 min. It was also effective in circulatory model. *V. cracca* extract at 200 mg/kg prolonged survival time (16.84 ± 1.47 min) that was statistically significant as compared to control group (13.14 ± 0.51 min). *V. cracca* extract showed a very good protective effect against the hypoxia in some models. Specifically, they produced significant and dose-dependent effect on the model of asphytic and haemic hypoxia. The presence of polyphenols in this plant may be a proposal mechanism for reported antihypoxic activities of this plant.

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Introduction

The inequality between oxygen demands and low oxygen supply leads to organ hypoxia. This condition occurs especially in ischemia, heart attack and heart diseases and causes many disturbances and finally resulting in death (1). In hypoxic condition, oxidative stress appears and production of reactive oxygen species (ROS) occurs. It is clear that compounds with antioxidant activity can scavenge ROS and are able to display antihypoxic property (1). There are increasing interests in using natural antioxidants instead of the chemical ones. Among the various medicinal plants, some endemic and edible species are of particular interest because they may be used for producing raw materials or preparations containing phyto-chemicals with significant antioxidant capacities and health benefits.

In tumor cells, amount of oxygen and nutrients is inadequate. Although this decrease in oxygen tension can be lethal for normal cells, but many tumor cells can survive under this hypoxic condition (2). Tumor cells in this situation are resistant to chemotherapy or radiation. Hypoxic conditions can lead to tumor progression and metastasis through different mechanisms (2). Hypoxic condition is frequently found in many tumor cells such as head and neck carcinoma and is known as a risk factor for prognosis (3). Therefore, antihypoxic agents can be regarded as anticancer agents, too. Altitude sickness, also known mountain sickness, is a pathological effect of high altitude on humans, caused by acute exposure to low partial pressure of oxygen at high altitude. Pulmonary and cerebral edema are symptoms which may threaten life. This sickness also leads to damage of lung and brain or organ failure (4). Antihypoxic agents can be regarded as a useful route in mountain sickness drug therapy, too. *Vicia* genus (Papilionaceae) has 45 species in Iran. The extract from *V. sativum* showed insecticidal activity. Also it has exhibited hepatoprotective effect against CCl_4 induced hepatotoxixity. Antioxidant activities of *V. faba*, *V. cracca* and *V. sativum* have been reported previously (5-7). Also anti-inflammatory and antinociceptive activity of *V. sativa*, *V. canescence* and *V. hirsuta* (8,9), antidepressant and antihemolytic activities of *V. sojakii* (10), protective effects of *V. hirsuta* against hypoxia-induced lethality in mice (11) and antimicrobial and cytotoxic activity of *V. faba* have been reported (5). Recently, aantioxidant and hepatoprotective properties of *V. cracca* against carbon tetrachloride induced oxidative stress in mice have been reported by our group (12). There is no report on antihypoxic activities of *V. cracca*. The aim of this study was to determine the antihypoxic activities of *V. cracca* aerial parts against hypoxia-induced lethality in order to understand the usefulness of this plant in treatment of ischemia.

Materials and Method

Animals

Male Swiss albino mice (22 ± 2 g) were randomly housed in groups of 10 in poly propylene cages at an ambient temperature, 25 ± 1 °C and 45-55% relative humidity, with a 12 h light: 12 h dark cycle (lights on at 7 a.m.). The animals had free access to standard pellet and water and libitum. Experiments were conducted between 8:00 and 14:00 h. All the experimental procedures were conducted in accordance with the NIH guidelines of the Laboratory Animal Care and Use. The Institutional Animal Ethical Committee of Mazandaran University of Medical Sciences also approved the experimental protocol.

Plant material and preparation of extract

V. cracca aerial parts were collected from Sari, Iran, in summer 2015. The sample was authenticated by Dr. Bahman Eslami, and the voucher specimen was deposited (No: 794) in the herbarium of the Biology School of Gaemshahr Azad University. Aerial parts were dried at room temperature. Dried materials were coarsely ground before extraction. 10 g of aerial parts was extracted at room temperature by percolation method using methanol. The extract was concentrated in a rotary evaporator until a crude solid extracts were obtained. The crude solid extracts were freeze-dried for complete solvents removal (yield: 13.5%) (12). Extract was standardized based on total phenol and flavonoid contents (12).

Asphyctic Hypoxia

Thirty-two mice were divided into four groups each containing eight mice. The animals were subjected to hypoxia by putting them individually in a tightly closed 300 ml glass container. The animals had convulsions and died from hypoxia. The latencies for death were recorded. The animals died approximately 2 min following convulsions. Mice received single i.p. injections of 100 and 200 mg kg-1 doses of extract or phenytoin (50 mg kg^{-1}) as 30 min before they were subjected to hypoxia. Another control group was treated with normal saline (13-15).

Haemic Hypoxia

Twenty-four mice were divided into three groups each containing eight mice. Control group was treated with normal saline. Thirty minutes after i.p. administration of 100 and 200 mg kg^{-1} doses of extract, $NaNO_2$ (360 mg kg-1) was applied i.p. to mice and antihypoxic activity was estimated as the latent time of evidence of hypoxia in minutes (13-15).

Circulatory Hypoxia

Twenty-four mice were divided into three groups each containing eight mice. The control group was treated with normal saline. Thirty minutes after i.p. administration of 100 and 200 mg kg-1 doses of extract, NaF (150 mg kg^{-1}) was applied i.p. to mice and antihypoxic activity was estimated in minutes as the latent time of evidence of hypoxia (13-15).

Statistical Analysis

GraphPad Prism 5 was used for Statistical Analysis. Data were presented as mean ± SD. Analysis of variance (ANOVA) was performed. Duncan's new multiple-range test was used to determine the differences in means. All p values less than 0.05 were regarded as significant.

Results

Total phenolic content was 17.2 ± 0.9 mg gallic acid equilavent (GAE) and flavonoid content was 9.78 ± 0.25 mg quercetine equivalent (QE) (12). Statistically significant antihypoxic activities were established in some doses of *V. cracca* in experimental models of hypoxia in mice. The results of asphytic hypoxia are shown in Figure 1. The effects were dose-dependent. *V. cracca* at two tested doses showed statistically significant activity respect to the control. At 200 mg kg^{-1}, it significantly prolonged the latency for death with respect to control group (27.37 ± 4.09 vs. 19.55 ± 3.19 min, $P < 0.01$). At 100 mg kg^{-1}, it also prolonged survival time (24.76 ± 3.72 min, $p<0.05$ respect to control). Phenytoin that used as positive control kept mice alive for 39.80 ± 1.92 min.

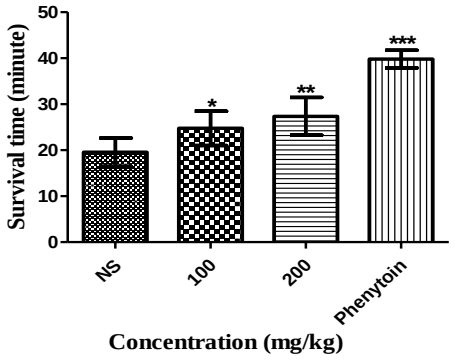

Figure 1 Antihypoxic activities of V. craccca in asphyctic hypoxia in mice. Data are expressed as mean ± SD (n = 8), (*P<0.05, **P<0.01, ***P<0.001, compared to control).

This effect was statistically significant from the control ($P < 0.001$). Extract showed good activity in haemic model (Fig. 2). Control group died of hypoxia in 11.90 ± 0.79 min. Extract at 200 mg kg^{-1} significantly prolonged latency for death with respect to control group (15.38 ± 1.93 min, $P < 0.01$). At 100 mg kg^{-1}, it also prolonged the latency for death with respect to control group (13.98 ± 1.654 min, $P < 0.05$ respect to control group). The results of circulatory hypoxia have been shown in figure 3. Extract at 200 mg kg^{-1}, prolonged the latency for death with respect to control group. This increase was statistically significant (16.84 ± 1.47 vs. 13.14 ± 0.51 min, $P < 0.01$). At 100 mg kg^{-1}, it also prolonged

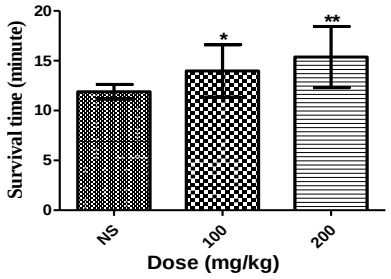

Figure 2 Antihypoxic activities of *V. cracca* in haemic hypoxia in mice. Data are expressed as mean ± SD (n = 8), (*P < 0.05, **P < 0.01, compared to control).

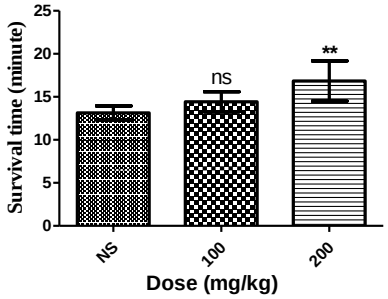

Figure 3 Antihypoxic activities of *V. cracca* in circulatory hypoxia in mice. Data are expressed as mean ± SD (n = 8). (ns; not significant, **P < 0.01, compared to control).

the latency for death with respect to control group but this increase was not statistically significant (14.43 ± 0.94 min, p>0.05).

Discussion

Hypoxia produces a strong physiologic stress and induces an extensive range of deleterious effects at the cellular level. The brain, which consumes a large quantity of oxygen, is very vulnerable to low levels of oxygen (16). Free radicals act as signaling species in various normal physiological processes but excessive production of these radicals causes damage to biological material. The increased level of ROS in hypoxia is the result of the accumulation of reduction equivalents in the mitochondrial electron transport system (17). The effects of ROS can be particularly evident in certain tissues such as brain because it consumes about 1/5 of the basal oxygen. Many efforts have been undertaken to develop therapies to reduce the effects of oxidative stress. Considerable evidence shows that antioxidants can protect a variety of illnesses. Polyphenols are powerful antioxidants and have a broad spectrum of pharmacological and therapeutic effects (18). *V. cracca* contains high level of polyphenoles (12). The survival time of animals in a sealed container directly reflects the antihypoxic activity.

Oxygen deficiency of the brain leads to deleterious changes in structural and functional integrity of cerebral tissue. Thus, any drug that enables the brain to resist the consequences of hypoxia or ischemia would be of great therapeutic interest (19). During the past decades a variety of different experimental models have been developed that could be used for testing antihypoxic and anti-ischemic drug effects in vivo (19). The brain is particularly affected by oxidative species because it has a high content of polyunsaturated fatty acids, which easily undergo oxidation.

Free radicals act as signaling species in various normal physiological processes but unnecessary production of these radicals causes harm to biological material. The increased level of ROS in hypoxia is the result of the accumulation of reduction equivalents in the mitochondrial electron transport system. The effects of ROS can be particularly evident in certain tissues such as brain. Many efforts have been undertaken to develop therapies to reduce the effects of oxidative stress. Considerable evidence shows that antioxidants can exert protecting action on a variety of illnesses.

The leaves and seeds of *V. cracca* are utilized as a forage crop for cattle and a nutritious food for pet birds. The cooked plant is galactogogue and the leaves are a tea substitute (20). Antioxidant activity of the ethanolic extracts of aerial parts of *V. cracca* (From Turkey) was screened by diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferric-reducing antioxidant power (FRAP) assays at 0.5, 1.0, and 2.0 mg ml^{-1} concentrations. Total phenolic contents of the extracts were determined using Folin-Ciocalteau reagent. In DPPH test, it showed weak activity at three concentrations. *V. cracca* was not tested in ferric-reducing power due to scarcity of its extract. No flavonoid was detected in aerial parts of *V. cracca* which may explain its low antioxidant effect (20). *V. cracca* showed weak inhibitions against DPPH radical at three concentrations (20). The IC50 value was 1.98 ± 0.25 mg ml^{-1} in our sample (12). Qualitative analysis of flavonoids; rutin, hyperoside, and vitexine by LC–DAD–MS was carried out in *V. cracca* extract. These three flavonoid-derivatives were not detected in extract (20).

Statistically significant antihypoxic activities were established in some doses of *V. cracca* in experimental models of hypoxia in mice (Figure 1). At 200 mg kg^{-1}, it significantly prolonged the latency for death with respect to control group (27.37 ± 4.09 vs. 19.55 ± 3.19 min, P < 0.01). *V. hirsuta* at 100 mg kg^{-1}, extract significantly prolonged the latency for death with respect to control group (51.00 ± 6.05 vs. 33.67 ± 4.50 min, P < 0.001) (21).

A close relationship between oxidative metabolism and cholinergic function has been found during the investigations of sodium nitrite on brain metabolism. Chemical hypoxia is induced by the injection of $NaNO_2$ (360 mg kg-1, i.p.), which reduces the oxygen-carrying capacity of the blood by converting hemoglobin to methemoglobin. This lethal dose is injected 30 min after the phenolic treatment. Immediately after the NaNO2 injection, the animals are placed in small cages

and the time between injection of $NaNO_2$ and cessation of respiration is recorded. As shown in figure 2, extract showed good activity in haemic model. Extract at 200 mg kg-1 significantly prolonged latency for death with respect to control group (15.38 ± 1.93 min, $P < 0.01$). In our recently published paper, control group died of hypoxia in 7.87 ± 0.78 min. V. hirsuta extract at 100 mg kg^{-1} significantly prolonged latency for death with respect to control group (15.60 ± 1.34 min, $P < 0.001$) (21).

There are literature data that administration of NaF, that induces circulatory hypoxia, increases the blood histamine content and decreases the oxygen carrying capacity (22). The results of circulatory hypoxia are shown in Figure 3. Extract at 200 mg kg-1, significantly prolonged the latency for death with respect to the control group (16.84 ± 1.47 vs. 13.14 ± 0.51 min, $p<0.01$). V. hirsuta extract at 100 mg kg-1, significantly prolonged the latency for death with respect to control group (14.83 ± 0.75 vs. 10.71 ± 1.12 min, $P < 0.001$) (21). It seems V. hirsuta showed more protective effect against the hypoxia in some model than our tested *V. cracca* (21).

Conclusion

V. cracca showed a very good protective effect against the hypoxia in some model. Specifically, they produced significant and dose-dependent effect on the model of asphytic and haemic hypoxia. The presence of polyphenols in this plant may be a proposal mechanism for reported antihypoxic activities of this plant.

Acknowledgments

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Conflict of Interest

Authors declare no conflict of interest in this study.

Author's Contribution

Dr Ebrahimzadeh designed the study. Dr Ebrahimzadeh performed the statistical analysis. Mrs Shahnazi did the experimental work. The first draft of the paper was written by two authors. All authors read and approved the final manuscript.

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