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Protective effects of ethanolic extract of Lemon Beebrush (Aloysia Citrodora) leaf against hypoxia-induced lethality in mice

Mohammad Hossein Hosseinzadeh,Mohammad Ali Ebrahimzadeh*

Student Research Committee and Pharmaceutical Sciences Research Center, Hemoglobinopathy Institute, Mazandaran University of Medical Sciences, Sari, Iran.

Lemon Beebrush, known as Lippia citroiodora and Aloysia Citrodora is a known medicinal plant in Iran. Many biological activities have been reported from this plant. Despite many works, nothing is known about protective effect of A. Citrodora against hypoxia conditions. In this study, protective effects of A. Citrodora leaf extract against hypoxia-induced lethality in mice were evaluated by three experimental models of hypoxia, asphyctic, haemic and circulatory. It's phenol and flavonoid contents and antioxidant activity were also evaluated. Statistically significant protective activities were established in some doses of extract in three models. Antihypoxic activity was especially pronounced in circulatory hypoxia where extract at 62.5 mg kg-1 prolonged the latency for death with respect to the control group ($p<0.01$). The effect was dose-dependent. At 250 mg kg-1, it prolonged the latency for death with the same activity of propranolol (20 mg kg-1), that used as positive control ($p>0.05$). Extract showed weak activity in haemic model. Only at the highest tested dose, 250 mg kg-1, it significantly prolonged latency for death with respect to control group ($p<0.05$). Extract at this dose showed the same activity of propranolol which used as positive control ($p>0.05$). In asphytic model, extract at the highest tested dose showed statistically significant activity respect to the control. At 250 mg kg-1, it significantly prolonged the latency for death (26.84 ± 4.11 vs. 19.45 ± 1.13 min, $p = 0.0006$). At 125 mg kg-1, it also prolonged survival time but this increase was not significantly different. Phenytoin that used as positive control kept mice alive for 29.60 ± 2.51 min ($p<0.0001$). Extract at 250 mg kg-1 showed the same activity of phenytoin ($p>0.05$). The total phenolic content was 342.9 ± 11.5 mg gallic acid equivalent/g of extract powder and flavonoid content was 90.2 ± 7.8 mg quercetin equivalent/g of extract powder. IC50 for DPPH radical-scavenging activity was 21.97 ± 2.4 mg/ml. The presence of polyphenols in this plant may be a proposal mechanism for reported antihypoxic activities.

Keywords: Antihypoxia, Asphyxia, Reactive Oxygen Species, Lemon Beebrush, Lippia Citroiodora, Aloysia Citrodora

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Introduction

Imbalance between oxygen demands and oxygen supply leads to organ hypoxia. This condition happens especially in heart attack, ischemia, heart diseases and causes many troubles and finally causing death [1]. In hypoxic condition, oxidative stress appears and production of reactive oxygen species (ROS) occurs. Antioxidants can scavenge these ROS and are able to show antihypoxic properties [1]. There are increasing interests in using natural antioxidants instead of the chemical antioxidants. Among the numerous medicinal plants, some of them are of particular interest because they may be used for preparation of phytochemicals with significant antioxidant capacities and health benefits.

In cancerous and tumor cells, amount of oxygen is inadequate. This reduction in oxygen level can be lethal for normal cells, but many cancerous and tumor cells can survive under this hypoxic condition. Tumor cells in this situation are also resistant to chemotherapy or radiation. It is known that hypoxia can lead to tumor progression and metastasis through different mechanisms [2]. The presence of hypoxia in some cancers such as head and neck carcinoma has been well established. This condition is known as a risk factor for prognosis.

*Corresponding author: **Mohammad Ali Ebrahimzadeh,** Student Research Committee And Pharmaceutical Sciences Research Center, Hemoglobinopathy Institute, Mazandaran University Of Medical Sciences, Sari, Iran. Email: Zadeh20@Yahoo.Com

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Therefore, antihypoxic agents can be regarded as anticancer agents, too. [3]

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 that may threaten life. This sickness also leads to damage to lung and brain or organ failure. Antihypoxic agents can be regarded as a useful route in mountain sickness drug therapy, too. [4]

The Lemon Beebrush, known as Lippia citroiodora Kunth H.B. et K and Aloysia Citrodora Orteg (Figure 1), is a flowering plant in Verbenaceae family [5]. In traditional medicine, the plant is used to treat insomnia, anxiety, gastrointestinal, respiratory, and cardiovascular problems. The leaves of this plant are rich in flavonoids, monoterpenes, and sesquiterpenes such as Nepetin, Geranial, and Citronella [6]. Recently excellent Neuroprotective effects of the essential oil of this plant have been reported [7]. The aqueous extract of this plant also has anxiolytic and hypnotic effects [8]. Trolox, Hypocholorities, DPPH, and FRAP tests at the invitro level have proven excellent antioxidant properties of this plant [9,10], supported by results from invivo studies. The effects of this plant on increasing cell resistance to oxidative stress, lipid peroxidation, and protein carbonylation have been confirmed [11,12]. Also, the ethanolic extract of this plant increased apoptosis of colon cancer cell line by increasing proapoptotic gene BAX activity and decreasing anti-apoptotic gene Bcl-2 [13].

Figure 1. Aerial parts of Aloysia Citrodora

There is no report on antihypoxic activities of Lemon Beebrush. The aim of this study was to determine the antihypoxic activities of Lemon Beebrush leaf against hypoxia-induced lethality in order to understand the usefulness of this plant in treatment of ischemia. In addition, its phenol and flavonoid contents and antioxidant activity were evaluated.

Materials and Methods

Animals

Male Swiss albino mice (25 ± 2 g) were randomly housed in groups of 10 in poly propylene cages at ambient temperature, 25 ± 1ºC 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

A. Citrodora leaf were collected from Sari, Iran, in summer 2019. The sample was authenticated by Dr. Bahman Eslami. Leaves were dried at room temperature. Dried materials were coarsely ground before extraction. 100 g of leaf was extracted at room temperature by maceration method using ethanol (95%) as solvent. The extract was concentrated in a rotary evaporator until a crude solid extract were obtained. The crude solid extracts were freeze-dried for complete solvents removal (yield: 3.9%) [14,15]. Extract was standardized based on total phenol and flavonoid contents [14,15].

Asphyctic Hypoxia

Thirty-five mice were divided into four groups each containing seven 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 62.5, 125 and 250 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 [16-18].

Haemic Hypoxia

Thirty-five mice were divided into three groups each containing seven mice. Control group was treated with normal saline. Thirty minutes after i.p. administration of 62.5, 125 and 250 mg kg-1 doses of extract, NaNO2 (360 mg kg-1) was applied i.p. to mice and antihypoxic activity was estimated as the latent time of evidence of

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hypoxia in minutes. Propranolol (20 mg kg-1) was used as positive control.

Circulatory Hypoxia

Forty-two mice were divided into three groups each containing seven mice. The control group was treated with normal saline. Thirty minutes after i.p. administration of 62.5, 125 and 250 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. Propranolol (20 and 40 mg kg-1) was used as positive control. [16-18].

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by Folin-Ciocalteau method. The extract (0.1 ml) was mixed with 0.5 ml of a 0.2 N Folin-Ciocalteau reagent for 5 min and then 0.4 ml of 75 g/L Na2CO3 were added. The absorbance of the reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated using AlCl3 colorimetric method. Briefly, 0.5 mL solution of extract in methanol were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% AlCl3, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). The total flavonoid contents were calculated as quercetin from a calibration curve.

DPPH radical-scavenging activity

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of the free radical-scavenging activity of the extract. Different concentrations of extract were added, at an equal volume, to a methanolic solution of DPPH (100 mM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated three times. BHA was used as standard controls. IC50 values denote the concentration of the sample, which is required to scavenge 50% of DPPH free radicals [14,15].

Statistical Analysis

GraphPad Prism 8 was used for Statistical Analysis. Data were presented as mean ± SD. Analysis of variance (ANOVA) was performed. Tukey multiple comparisons test was used to determine the differences in means. All P-values less than 0.05 were regarded as significant.

Results

The total phenolic content was 342.9 ± 11.5 mg gallic acid equivalent/g of extract powder in reference to the standard curve (y = 0.0054x + 0.0623, r2 = 0.997). The total flavonoid content was 90.2 ± 7.8 mg quercetin equivalent/g of extract powder, in reference to the standard curve (y = 0.0064x+ 0.0076, r2 = 0.999). IC50 for DPPH radical-scavenging activity was 21.97 ± 2.4 µg/ml. The IC50 value for BHA was 53.96 ± 3.16 µg/ml.

Statistically significant antihypoxic activities were established in some doses of A. Citrodora in experimental models of hypoxia in mice. The results of asphytic hypoxia are shown in Figure 2.

Extract at the highest tested dose showed statistically significant activity respect to the control. At 250 mg kg-1, it significantly prolonged the latency for death with respect to control group (26.84 ± 4.11 vs. 19.45 ± 1.13 min, p = 0.0006). At 125 mg kg-1, it also prolonged survival time but this increase was not significantly different. Phenytoin that used as positive control kept mice alive for 29.60 ± 2.51 min. This effect was statistically significant from the control (p<0.0001). Extract at 250 mg kg-1 showed the same activity of phenytoin (p>0.05).

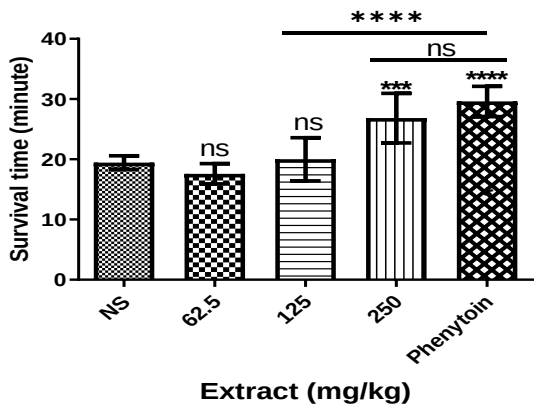

Figure 2. Antihypoxic activities of A. Citrodora in asphyctic hypoxia in mice. Data are expressed as mean ± SD (n = 7), (ns, P>0.05, ***P=0.0006, ****P<0.0001).

Extract showed weak activity in haemic model (Figure 3). Control group died of hypoxia in 12.12 ± 0.77 min. Extract at 250 mg kg-1 significantly prolonged latency for death with respect to control group (14.47 ± 1.24 min, p<0.05). In this test, propranolol was used as positive control. Propranolol at 20 mg kg-1 prolonged the latency for death with respect to control group (16.44 ± 1.39 min, p<0.001 respect to control group). Extract at 250 mg kg-1 showed the same activity of propranolol (P>0.05).

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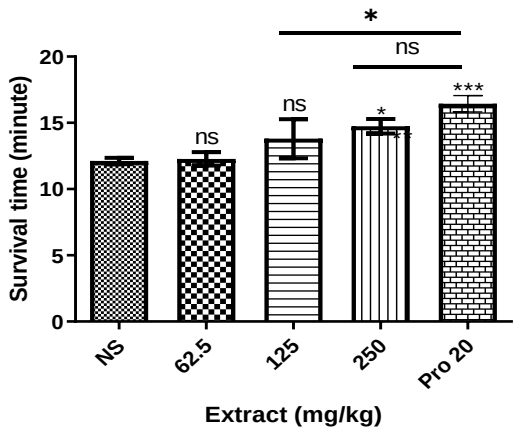

Figure 3. Antihypoxic activities of A. Citrodora in haemic hypoxia in mice. Data are expressed as mean ± SD (n = 7), (ns; not significant, *P<0.05, ***P<0.001).

The results of circulatory hypoxia have been shown in Figure 4. Extract showed high activity in test. At 62.5 mg kg-1, it prolonged the latency for death with respect to control group. This increase was statistically significant (13.17 ± 1.93 vs. 10.26 ± 1.30 min, p<0.01). The effect was dose dependent. At higher dose (125 mg kg-1), the better activity was observed (15.08 ± 2.21, p<0.001, respect to the control group). At 250 mg kg-1, it prolonged the latency for death with the same activity of propranolol (20 mg kg-1), that used as positive control (17.12 ± 1.76 vs. 16.44 ± 1.39 min, p>0.05).

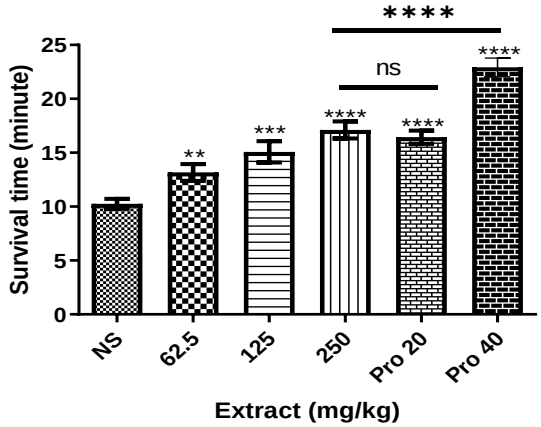

Figure 4. Antihypoxic activities of A. Citrodora in circulatory hypoxia in mice. Data are expressed as mean ± SD (n = 7). (ns; not significant, **P<0.01, ***P<0.001, ****P<0.0001).

Discussion

Hypoxia is defined as a decrease in available oxygen reaching the body tissues that can lead to impairment of body function and may cause a variety of physiological abnormality. It is linked to the pathology of acute mountain sickness, cardiovascular disease, and stroke, the leading causes of death in many countries [19].

Free radicals act as signaling species in various normal physiological processes but excessive production of these radicals causes damage to biological material [20]. The effects of ROS can be particularly evident in certain tissues such as brain because it consumes about 20% of the basal oxygen. Many efforts have been undertaken to develop therapies to reduce the effects of oxidative stress. Evidence shows that antioxidants can protect a variety of illnesses. Polyphenols are powerful antioxidants and have a broad spectrum of pharmacological and therapeutic effects [21].

Oxygen deficiency of the brain leads to deleterious changes in functional and structural integrity of cerebral tissue. Therefore, any compound that enables the brain to resist the consequences of hypoxia or ischemia would be of great therapeutic interest. 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 [22].The brain is particularly affected by oxidative species because its polyunsaturated fatty acids can easily undergo oxidation.

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 apparent 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.

Statistically significant antihypoxic activities were established in some doses of A. Citrodora in experimental models of hypoxia in mice (Figure 2). At 250 mg kg-1, it significantly prolonged the latency for death with respect to control group (p = 0.0006). at this dose, A. Citrodora showed the same activity of phenytoin (p>0.05).

Lack of oxygen in environment can lead to low oxygen partial pressure in mitochondria which made the cell die without enough energy. However, chemical intoxicant hypoxia model in mice induced by sodium nitrite was not the same. Sodium nitrite cut off the respiratory chain that caused the cell to be unable to use oxygen in the production of energy. Chemical hypoxia is induced by the injection of NaNO2 (360 mg kg-1, i.p.), reduces the oxygen-carrying capacity of the blood by converting hemoglobin to methemoglobin. [23] 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

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injection of $NaNO_2$ and cessation of respiration is recorded. As shown in Figure 3, extract showed weak activity in this model. Extract at 250 mg kg-1 showed the same activity of propranolol (250 mg kg-1) which used as positive control ($p>0.05$). 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, $p<0.001$) [24].

There are literature data that administration of NaF, that induces circulatory hypoxia, increases the blood histamine content and decreases the oxygen carrying capacity [17,18]. The results of circulatory hypoxia have been shown in Figure 4. Extract showed high activity in test. At 62.5 mg kg-1, it prolonged the latency for death with respect to control group. This increase was statistically significant (13.17±1.93 vs. 10.26±1.30 min, $p<0.01$). The effect was dose dependent. At higher dose (125 mg kg-1), the better activity was observed (15.08 ± 2.21, $p<0.001$, respect to the control group). At 250 mg kg-1, it prolonged the latency for death with the same activity of propranolol (20 mg kg-1), that used as positive control (17.12 ± 1.76 vs. 16.44 ± 1.39 min, $p>0.05$). Based on our recently published paper, V. hirsuta also showed strong protective effect against the hypoxia in this model [24].

Total phenol compound, as determined by the Folin Ciocalteau method, was reported as gallic acid equivalents and total flavonoid content was reported as the quercetin equivalent/g of extract powder by $AlCl_3$ colorimetric method. This plant showed high total phenol and flavonoid contents.

Based on the findings of the current study, 83.4 Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and they have been shown to possess significant antioxidant activities [25]. Studies have shown that increasing the flavonoids content in the diet could decrease some human diseases [14,15]. The model of scavenging the DPPH radical is a broadly used for evaluating free radical scavenging ability of many samples. DPPH is a stable nitrogen-centered free radical, the color of which turn to yellow upon reduction by either the process of hydrogen or electron donation. Substances which are able to perform this color change, can be considered as antioxidants and radical scavengers [14,15]. It was found that the radical- scavenging activity of the extract increased with increasing concentration. IC50 for DPPH radical-scavenging activity was 21.97 ± 2.4 µg/ml. The high total phenol and flavonoid contents of this plant may lead to its good DPPH-scavenging activity.

The vasculature within most solid tumours consists of abnormally formed, poorly functional blood vessels that are incapable of delivering sufficient oxygen and nutrients to properly support the growing tumour mass [26]. Although reduced oxygen level can be lethal for some cells, many tumour cells are able to survive under hypoxic conditions. It is well-established that hypoxic tumour cells are resistant to radiation therapy. Hypoxia stimulate tumour metastasis through a variety of direct and indirect mechanisms, and hypoxic tumour cells, therefore, represent a significant impediment to successful cancer therapy [6]. It seems good antihypoxic activity of this extract can justify its reported anticancer activity [13]. Many papers about medicinal plants (such as Hibiscus esculentus seeds, Sambucus ebulus leaf and fruit and Myrtus communis leaf and Allium sativum flower) with high antihypoxic activities have been published by our group [16, 27, 28].

Conclusion

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

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Conflicts of interest

Authors declare no conflict of interest in this study.

Author's Contribution

E. MA. designed the study and performed the statistical analysis. H. MH. 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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