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ORIGINAL: Effects of Methanolic Extract of *Ginkgo biloba* Leaf against Hypoxia-Induced Lethality in Mice

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ABSTRACT

Introduction: Hypoxia defines as a condition in which body tissues do not take sufficient oxygen supply. Chronic hypoxia has various medical consequences. Recently, the role of hypoxia in the progression of COVID-19 disease has been proven. *Ginkgo biloba* is a valuable plant from more than 2000 years ago. Ginkgo has antioxidant activity and exhibits good scavenging activity on the free radicals therefore, it is considered helpful in treating diseases associated with the generation of free radicals, including chronic inflammation, cerebral infarction, ischemic heart disease, and aging.

Material and Methods: In this study, anti-hypoxic activities of *G. biloba* methanolic leaf extract have been determined against hypoxia-induced lethality in mice to understand its usefulness in treating ischemia.

Results: The extract showed weak activity in asphyctic model. At 125 mg/kg, it significantly delayed the time of death compared to the control group ($p<0.05$) but did not show any activities in haemic or circulatory hypoxia tests even at a higher tested dose, 250 mg/kg. Although, at this dose, extract prolonged the survival time more than 1 minute in circulatory model, but this increase was not statistically significant.

Conclusion: In conclusion, results from this study showed that extract has weak anti-hypoxic effects in the treatment of hypoxia.

Introduction

Hypoxia defines as a condition in which body tissues do not take sufficient oxygen (O_2) supply. Maintaining cellular function will be affected by an imbalance between tissue O_2 supply and consumption, resulting in insufficient O_2 (1, 2). Chronic hypoxia has various medical consequences, including chronic obstructive lung disease, pulmonary hypertension, Eisenmenger's syndrome, polycythemia, and weight loss, all of which are associated with significantly elevated mortality (3). As a potent microenvironmental agent inducing metastatic tumor progression, hypoxia is correlated with poor survival in various cancer patients. Hypoxia straight increases the expression of genes involved in angiogenesis, glycolysis, immune suppression, invasion, and the cancer stem cell phenotype (4). Also, the role of hypoxia in the progression of COVID-19 disease has been proven (5).

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Ginkgo biloba L.(Syn.: Salisburia biloba Hoffmag,Salisburia adiantifolia, -Common names: ginkgo, maidenhair-tree), has been around for about 280 million years (6, 7), regarded as a valuable plant for humankind for more than 2000 years and is recognized as a "living fossil". Though its natural habitat is in Japan, Korea, and China, It seems that Zhejiang's remote mountainous valleys in eastern China are the plant's primary origin (8). It is considered the just surviving tree species of the order Ginkgoales. for several hundred years. *G. biloba* contains different components such as proanthocyanidins, flavonoids, ginkgolic acids, ginkgotoxins, terpene trilactones, polyflavones, biflavone (8, 9). Variations in the different plant components are primarily related to harvesting stages, drying process, and storage (10). Ginkgo extract has antioxidant activity and exhibits an extreme scavenging activity on free radicals (11); therefore, it is considered helpful in treating diseases associated with the generation of free radicals, including chronic inflammation, cerebral infarction, ischemic heart disease, and aging (12). Numerous studies conducted both in vitro and in vivo with experimental animals and humans have reported that 'EGb 761', an extract of the leaves of *G. biloba*, and some of its components may also have anti-cancer (chemopreventive) activities (13, 14). It seems that EGb 761, acting as antioxidants, can reverse the harmful effects of oxidative damage generated by free radicals and related ROS (15, 16).

As studies above have shown, hypoxia has played a vital role in the pathogenesis of these diseases, and ginkgo extract has shown significant therapeutic effects. Antioxidants have potentially Antihypoxic activities. It can be suggested that ginkgo has a therapeutic effect on these diseases due to its anti-hypoxia effects. This study aimed to ascertain the anti-hypoxic activities of *G.biloba* leaf against hypoxia-induced lethality to understand its usefulness in treating ischemia. Furthermore, its antioxidant activity and phenol and flavonoid contents were assessed.

Methods

Plant material and preparation of extract

G. biloba leaves were purchased from the local market. Dr. Bahman Eslami authenticated the sample. Leaves were dried and powdered. 10 g of plant powder was soaked in 70 ml of methanol. After 24 hours, the solvent was filtered. The extraction was repeated three times. The solvents were collected and finally dried by a rotary evaporator (at 35 °C) and then freeze-dried until a solid crude extract was obtained (yield: 12.5%) (17).

Determination of total phenol contents

One-half ml of extract (0.5 mg/ml) was prepared. It was mixed with 2.5 ml of Folin-Ciocalteau solution (0.2 N) and 2 ml of sodium carbonate (75 g/L). After 2 hours, the adsorption of the solution was measured at 760 nm. Also, different gallic acid concentrations were prepared. Their absorption was evaluated based on the same method, and the standard curve was drawn. Finally, the total phenol content was reported based on the gallic acid equivalent /g of extract (17).

Determination of total flavonoid contents

One ml of extract (1 mg/ml) was prepared. It was mixed with 3 ml of methanol, 0.2 ml of aluminum chloride (10%), 0.2 ml of potassium acetate (1M), and 5.6 ml of distilled water. After 30 minutes, the adsorption of the solution was measured at 415 nm. Also, different quercetin concentrations were prepared. Their absorption was evaluated based on the same method, and the standard curve was drawn. Finally, the total flavonoid content was reported based on the quercetin equivalent /g of extract (17).

Animals

Male Swiss albino mice (27.8±2.2 g) were randomly housed in 26 ± 1ºC temperature and 45-55% relative humidity (12 h light: 12 h dark cycle, lights on at 7 a.m.). The animals had free access to standard pellet, water, and libitum. All the experimental procedures

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were approved by the Institutional Animal Ethical Committee of Mazandaran University of Medical Sciences (IR.MAZUMS.REC. 1398.1448).

Asphyctic Hypoxia

Thirty mice were randomly divided into five groups. The groups were exposed to i.p. injection of normal saline, extract with doses of 62.5, 125, 250, and phenytoin (50 mg/kg), respectively. After 30 minutes, the mice were placed in a tightly closed 300 ml glass container. The latencies for death were measured as an asphyctic hypoxia inhibition factor (18).

Haemic Hypoxia

Thirty mice were randomly divided into five groups. The groups were exposed to i.p. injection of normal saline, extract with doses of 62.5, 125, 250, and Propranolol (20 mg/kg), respectively. After 30 minutes, sodium nitrate (360 mg/kg) was injected peritoneally. The latencies for death were measured as a haemic hypoxia inhibition factor (18).

Circulatory Hypoxia

Thirty mice were randomly divided into five groups. The groups were exposed to i.p. injection of normal saline, extract with doses of 62.5, 125, 250, and Propranolol (30 mg/kg), respectively. After 30 minutes, sodium fluoride (150 mg/kg) was injected peritoneally. The latencies for death were measured as a circulatory hypoxia inhibition factor (18).

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

Total phenol and flavonoid contents

Total phenolic content of *G. biloba* leaves was 167.79±5.04 mg gallic acid equivalent/g of extract ($y = 0.0054x + 0.0623$, $R^2 = 1$) and total flavonoid content of *G. biloba* leaves was 93.21±1.41 mg quercetin equivalent/g of extract ($y = 0.0064x - 0.0076$, $R^2 = 0.9998$).

Antihypoxic effects

The results of hypoxia tests are shown in ***Table 1***. *G. biloba* leaf extract only showed weak activity in the asphyctic model. At 125 and 250 mg/kg, it significantly delayed the time of death compared to the control group ($p<0.05$), but at a lower dose. i.e. 62.5, it showed any effect ($p>0.05$). Phenytoin, which was used as a positive control group, showed a very powerful effect, where prolonged survival time from 19.05±2.31 for the control group to 29.60±2.51 min. ($p< 0.0001$).

The extract did not show any activities in haemic or circulatory hypoxia tests even at a higher tested dose, 250 mg/kg. Although, at this dose, extract prolonged the survival time more than 1 minute in circulatory model, but this increase was not statistically significant. In these two tests, propranolol was used as a

Table 1. Anti-hypoxic activities of G. biloba leaf extract in asphyctic, haemic, and circulatory hypoxia models in mice

Groups	Dose (mg/kg)	Asphyctic Hypoxia	Haemic Hypoxia	Circulatory Hypoxia
Control (NS)	-	19.05±2.31	10.50±0.66	10.11±0.86
Extract	62.5	21.62±0.92^{ns}	9.49±0.67^{ns}	10.05±0.91^{ns}
	125	22.59±1.18*	9.71±0.54^{ns}	10.06±2.27^{ns}
	250	22.80±1.48*	9.86±1.73^{ns}	11.13±2.45^{ns}
Phenytoin	50	29.60±2.51****	-	
Propranolol	20	-	16.48±1.98****	-
	30	-	-	15.91±1.77***

Data are expressed as mean ± SD (n = 6), (ns, not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, compared to control).

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positive control group. It was effective in both tests (***Table 1***).

Discussion

The role of hypoxia as a critical factor in pathogenesis has been proven in several conditions such as polycythemia, cardiopulmonary disorders, and COVID-19 (3,5,19). The sustained hypoxia in a growing tumor may induce cellular changes resulting in a more clinically aggressive phenotype (20, 21). Tumors may extend an enhanced potential for local invasive growth, regional and distant tumor cell spreading, Through the process of hypoxia-driven malignant progression (22). Furthermore, chronic hypoxia may increase intrinsic resistance to radiation and other cancer therapies (22, 23).
Numerous herbal compounds have been shown to have notable anti-hypoxia effects that could be used as a potential treatment for a broad range of diseases (24, 25). Recently, good antihypoxic activities were reported for dexamethasone as an effective anti COVID-19 drug in the clinic (26). Other drugs such as Magnesium sulfate (27) or Edaravone (28) and some medicinal plants with high antihypoxic activities such as *Lemon Beebrush* (29), *Allium sativum* (30), *Crataegus spp*. (31), *Juglans regia* (32) and *Sambucus ebulus* (33) or *Cantharellus cibarius* (34) are good candidates for the treatment of COVID-19.
In the haemic hypoxia test, sodium nitrite is used to induce blood poisoning, in which the carrier factors, hemoglobin, bind to the substance with greater affinity and a stronger bond. However, hemoglobin is not structurally abnormal in the mitochondria of energy production chains. Therefore, the binding of circulating oxygen to hemoglobin is prevented, and as a result, oxygen delivery becomes difficult. In chemical poisoning using sodium nitrite, the oxygen-carrying capacity is reduced by converting hemoglobin to methemoglobin. As a result, hypoxia in tissue cells causes the death of an organism (35). Findings of the study revealed that in haemic hypoxia, the extract has no observable therapeutic effect in any tested doses (up to 250 mg/kg).
In the circulatory poisoning, sodium fluoride causes hemoglobin to lysis. As a result, the cell's oxygen-carrying capacity is reduced, leading to hypoxia and death. Sodium fluoride in high concentrations induces acute poisoning and hemoglobin breakdown, resulting in the entry of compounds into its structure into the bloodstream. In the circulatory hypoxia test, if a positive effect is obtained in increasing mice's survival time, the reason may be mentioned in better oxygenation due to decreased hemoglobin lysis in the blood or increased cell resistance to hypoxia (35). The results of this study exhibited that in circulatory hypoxia, the extract has no significant therapeutic effect in any tested doses (up to 250 mg/kg).
The asphyxia hypoxia model is one of the models that simulate oxygen deficiency conditions in the cell. In this test, phenytoin was used as a positive control. It reduces cellular activity, oxygen consumption, and ATP and increases resistance to hypoxia (26). In the asphyctic hypoxia test, the study finding shows that *G. biloba* extract at higher doses (125 and 250 mg/kg) delayed the death time significantly (about 3 minutes), but at a lower dose (62.5 mg/kg) did not show any activity.
In short, it can be inferred that *G. biloba* methanolic leaf extract has very weak potential therapeutic effects in treating hypoxia, however, its main constituents should be tested for better activities in these models.

Conclusion

Although good effects of *G. biloba* extract have been reported in the treatment of hypoxia-related diseases, the results of this study showed that the plant extract has weak anti-hypoxic effects in the treatment of hypoxia. Methanolic extract of *G. biloba* showed weak effect in the asphyctic hypoxia test. In haemic and circulatory hypoxia tests, it did not improve the survival times.

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Conflicts of interest

The authors declare no conflict of interest in this study.

Authors' contributions

All authors designed the study. E. MA. performed the statistical analysis. H. MH, and M. P. did the experimental works. The first draft of the paper was written by all authors. All authors read and approved the final manuscript.

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