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# Estimation the Total Flavonoid, Reductive Ability, Free Radical Scavenging Potentials and Reactive Oxygen Species Reduction of *Ginkgo biloba* Ethanolic Extract

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## Abstract

Oxidative stress is a pathological condition characterized by an imbalance between the production of reactive oxygen species and the body's antioxidant defenses. This imbalance can lead to cellular damage and has been implicated in various disease states. *Ginkgo biloba*, a widely studied medicinal plant, is known to possess potent antioxidant properties due to its phytochemical composition. This study aimed to comprehensively evaluate the antioxidant activity and protective effects of *G. biloba* ethanolic extract (GB-EE). The total flavonoid content of the extract was determined, and its free radical scavenging ability and reducing power were assessed. GB-EE exhibited a concentration-dependent DPPH radical scavenging activity, with an $IC_{50}$ of 19.45 µg/mL, comparable to the positive control, ascorbic acid (26.35 µg/mL). Additionally, the extract demonstrated moderate reduction ability, as evidenced by its ferric-reducing antioxidant power. Furthermore, *in vivo* studies on mice revealed that GB-EE treatment protected against hydrogen peroxide-induced oxidative stress, significantly reducing malondialdehyde levels and restoring glutathione concentrations in the kidney and liver tissues. These findings suggest that GB-EE possesses potent antioxidant properties and can exert a protective effect against oxidative damage, highlighting its potential therapeutic applications.

**Keywords:** *Ginkgo biloba*, Antioxidants, DPPH assay, FRAP assay, Oxidative Stress.

## Introduction

For centuries, medicinal plants have been an essential resource and remain a mainstay in traditional medicine for treating local ailments of the indigenous people [1]. The World Health Organization (WHO) estimates that 88% of member countries use herbal plants, acupuncture, yoga, and local remedies in traditional medicine to treat some local diseases [2]. Medicinal plants represent major resources of natural

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products that have the ability to fight many serious diseases [3]. *Ginkgo biloba* is the oldest living tree with a long history of use as a therapeutic plant in traditional Chinese medicine [4]. Several studies showed that leaf extracts of *G. biloba* have significant contents of phytochemicals products like flavonoids (24%), terpenoids (6%), and natural antioxidants ginkgolic acids (less than 5 ppm) [5].

Flavonoids are secondary metabolites in plants and can be divided into flavones, flavonols, dihydroflavonols, dihydroflavones, flavanones and isoflavones [6]. More than 5000 flavonoids have been found in edible plants. Dietary flavonoids possess multiple bioactivities, including antioxidative, anti-obesity and antiadipogenic effects. However, flavonoids as natural antioxidants have the ability for reducing (ROS) in human bodies [7].

Reactive oxygen species (ROS) are highly reactive due to the presence of unpaired electrons [8]. In case of oxidative stress, ROS that occurred during aerobic metabolic activities such as superoxide, hydroxyl, nitric acid, hypochloric acid, proxynitrite and peroxyl radicals play an essential role in the pathogenesis of various serious diseases [9]. Oxidative damage by free radicals has been considered as a mechanism involved in the pathogenesis of many chronic diseases such as atherogenesis and other heart diseases [10], in cancer [11], neural disorders [12], and it also play an adverse effect on the female reproductive functions [13]. ROS significantly impacts reproductive function by inducing oxidative stress, damaging cellular components like DNA, proteins, and lipids [14]. Therefore, reducing the rates of ROS in the bodies to the lowest levels became extremely required.

Since dietary plants contain most antioxidant compounds that are consumed by human bodies, it is essential to find methods that can measure the total flavonoids of plant extracts directly [15]. In the current study, total flavonoids and antioxidant activities were evaluated in *G. biloba* ethanolic extract through DPPH Radical - Scavenging Activity (1,1-diphenyl-2-picrylhydrazyl) and Ferric-reducing antioxidant power (FRAP) assays and examine its alleviating effect *in vivo*.

## Materials and Methods

### Plant Collection and Identification

Dried *G. biloba* leaves used in this study were obtained from local markets of Baghdad-Iraq which previously identified by National Herbarium of Iraq. The plant's leaves were thoroughly washed in tap water to remove impurities, dried and grinded into fine powder using electrical grinder.

### Preparation of *G. biloba* Ethanolic Extract (GB-EE)

This method was carried out according to [16]. The plant ethanolic extract was prepared by adding 50 g of leaves powder in 250 mL of ethanol (99%). Then the mixture was incubated in shaker-incubator at 37°C for 24 hours. At first, the mixture was filtered by multiple layers of gauze followed by Whatmans filter paper No.1. In addition, the filtrate was concentrated well by rotary evaporator. Stock solution was perpared, then different concentrations of the extract were prepared 25, 50,100, and 200 µg/mL.

### Determination of Total Flavonoid Content

The aluminum chloride colorimetric method which was described by [17] was used to evaluate the total flavonoid content Spectro-photosynthetically. Briefly, 3.5 mg of *G. biloba* ethanolic extract was

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dissolved in 5 mL of half ethanol, followed by addition of 1 ml of 5% (w/v) NaNO2 solution. After 6 min, 1 mL of a 10% (w/v) $AlCl_3.6H_2O$ solution was added and the mixture was left for a further 5 min before adding 10 mL of a 10% (w/v) NaOH and the mixture was made up with 50 mL of distilled water and mixed well. Furthermore, the absorbance was evaluated using a spectrophotometer and it was 460 nm. A linear standard curve of catechin was prepared. Total flavonoid was calculated based on standard curve equation y =3.25x + 0.019, where y = absorbance at 460 nm and x = flavonoid concentration. The obtained result was expressed as means (SD) mg of (+)-catechin equivalents per gramme gram for the *G. biloba* ethanolic extract.

**DPPH Radical - Scavenging Activity**

The measurement of the antioxidant activity of *G. biloba* ethanolic extract and the standard were done according to the radical–scavenging effect of the stable 1,1 diphenyl-2-pecrylhdrazyl (DPPH) free radical method [18]. An aliquot of 0.1 mL of *G. biloba* ethanolic extract or standard (Ascorbic Acid) (25, 50, 100 and 200 µg/mL) was added to 3.9 mL of DPPH solution in a test tube. And the mixture was incubated in dark at 37°C for 30 min. In addition, spectrophotometer apparatus was used to determine the absorbance of each mixture at 517 nm. All measurements were performed in triplicates. The ability to scavenge DPPH free radical was calculated by the equation given below:

$$(DPPH)\ Radical\ Scavenging\ Activity\ (\%) = \left(1 - \frac{Absorbance\ of\ Sample}{Absorbance\ of\ Standard}\right) \times 100$$

**Reductive Ability**

To determine the reductive ability of *G. biloba* ethanolic extract, the method that described by [19] was adopted. One mL of each concentration of *G. biloba* ethanolic extracts (25, 50, 100 and 200 µg/mL) or standard (Trolox) was added to 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1.5 mL of potassium ferricyanide (1%), and the mixture was incubated at 50°C in dark for 20 min. One mL trichloroacetic acid (10%) was added to the obtained solution to stop the interaction. After that, the mixture was centrifuged at 3000 rpm for 10 min. Then 2 mL of distilled water and 0.5 mL of freshly prepared ferric chloride (0.1%) was mixed with 2.5 mL of the obtained supernatant. Furthermore, the absorbance was measured at 700 nm with a spectrophotometer after 15 min and all tests were carried out in triplicate.

**Experimental Animals**

BALB/c male mice, approximately 30 g (12 to 14 weeks old) with total number of 40, were used in this study and kindly provided from Biotechnology Research Center – Al-Nahrain University. Mice were housed in stainless steel cages, under a 12-hour dark/12-hour light cycle at a temperature of 22±3.0°C. The investigation was conducted at a relative humidity of 55±5.0%. Mice fed water and conventional pellet meal throughout the study period.

Mice were separated into 4 groups, each group contained 10 mice as follows:

Group 1 (Untreated mice): mice were administrated with 0.1 mL PBS intraperitoneally for 14 days.

Group 2 (GB-EE Treatment): mice were intraperitoneally injected with 0.1 mL of 100 mg/kg GB-EE for 14 days.

Group 3 ($H_2O_2$ Treatment): mice were intraperitoneally injected with 0.1 mL of 100 mg/kg $H_2O_2$ for 14 days.

Group 4 (GB-EE + $H_2O_2$ Treatment): mice were injected with 0.1 mL (100 mg/kg) GB-EE for 5 days prior to dual H2O2 and GB-EE treatment (100 mg/kg for each) for 9 days.

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At the end of the experiment, all mice were sacrificed, kidney and liver tissues were collected and homogenized in cold KCl 0.15 M using homogenizer (15,000 rpm). The homogenates underwent centrifugation, and the supernatants were preserved at -40°C until analysis. Tissue malondialdehyde (MDA) and glutathione (GSH) concentrations were quantified using a UV-spectrophotometer employing the colorimetric techniques as previously reported [20].

**Data Analysis**

All values in this study are given as mean ± standard deviation (SD), and differences between means were determined by analysis of variance (ANOVA) followed by either LSD (Least Significant Difference) of Duncan's test. The analyses were achieved by using the statistical package SPSS version 20.

## Results and Discussion

**Total Flavonoids Content**

Ethanolic extract of *G. biloba* was found to have total flavonoids of 832.1±15.8 µg/mL. Accordingly, the ethanolic extract of *G. biloba* can be considered as a rich source of flavonoids. Phenolic compounds with variable structures are the main components of flavonoids and can be found in fruits, vegetables, grains, bark, roots, stems and flowers which in turn belong to a wide-group of plant secondary metabolites. Moreover, Flavonoids have a wide range of therapeutic activities including their potential function as anti-cancer agents which give them a remarkable therapeutic feature [21, 22].

**DPPH Radical Scavenging Activity**

The radical scavenging activity of GB-EE was estimated by comparing the percentage inhibition of formation of DPPH radical by the extract with those of ascorbic acid. GB-EE exhibited a dose-dependent antioxidant capabilities with maximum scavenging of DPPH was 76.23±2.022% at 200 µg/mL. Compared to ascorbic acid (as a positive control), the pattern of GB-EE scavenging activity showed no significant differences with ascorbic acid treatment, indicating the substantial antioxidant activity of GB-EE. The calculated $IC_{50}$ was 19.45 and 26.35 µg/mL for GB-EE and ascorbic acid, respectively (Table 1).

**(Table 1): DPPH radical scavenging activity of GB-EE.**

| Treatment (µg/mL) | Antioxidant Activity (Mean ± SD) % | | |
|---|---|---|---|
| | Ascorbic acid | GB-EE | *p* Value |
| **25** | 49.04±0.8839 | 38.46±0.4064 | 0.9998 NS |
| **50** | 53.86±1.381 | 57.56±11.27 | 0.7840 NS |
| **100** | 69.48±4.291 | 60.88±1.252 | 0.1123 NS |
| **200** | 76.23±2.022 | 73.15±1.140 | 0.8733 NS |

**NS: Non-significant.**

GB-EE demonstrates notable antioxidant properties, attributed to its abundant bioactive compounds, especially flavonoids, terpene lactones, and polyphenolic compounds. The antioxidant activity of the extract is mainly due to its capacity to neutralize free radicals, inhibit lipid peroxidation, and safeguard cells from damage caused by oxidative stress [23]. The principal flavonoid constituents play a

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crucial role in enhancing antioxidant capacity via mechanisms including hydrogen atom donation and electron transfer [24].

**Reductive Ability Assay**

In this experiment, the assessment of the reductive ability showed that the GB-EE was moderate in such activity, in which the obtained results were concentration-related. GB-EE exhibited efficient reduction ability for all concentrations used with significant differences ($p < 0.01$) compared to trolox (positive control). At the concentration 0.64 mg/mL of GB-EE the highest absorbance was observed in comparison with that of trolox and the absorbance values were 0.4±0.01 and 0.278±0.01 respectively (Table 2).
The moderate reduction activity of the extract is evidenced by its ability to reduce ferric ($Fe^{3+}$) to ferrous ($Fe^{2+}$) ions, as measured by the FRAP (Ferric Reducing Antioxidant Power) assay. This reducing power, while not as potent as its antioxidant capacity, plays a crucial role in maintaining cellular redox homeostasis. The synergistic interaction between the extract's reducing and antioxidant properties enhances its overall therapeutic potential, particularly in conditions characterized by oxidative stress and redox imbalance [25].

**(Table 2): Reductive ability of GB-EE.**

| Treatment (mg/mL) | Absorbance (Mean ± SD) | | |
|---|---|---|---|
| | Positive Control (Trolox) | *G. biloba* | *p Value* |
| **0.02** | 0.109±0.002 | 0.179±0.003 | <0.0001 ** |
| **0.04** | 0.117±0.002 | 0.234±0.005 | <0.0001 ** |
| **0.08** | 0.138±0.008 | 0.337±0.007 | <0.0001 ** |
| **0.16** | 0.164±0.015 | 0.361±0.007 | <0.0001 ** |
| **0.32** | 0.230±0.022 | 0.390±0.008 | <0.0001 ** |
| **0.64** | 0.278±0.010 | 0.400±0.010 | <0.0001 ** |

****: $p < 0.01$.**

**GB-EE Exhibited Protective Effect Against $H_2O_2$ Toxicity**

Results in Fig. (1) and Table (3), show that the level of MDA and GSH in mice treated with GB-EE was close to control group, however, $H_2O_2$ treatment resulted in extreme changes at these levels. In $H_2O_2$ treated mice, the level of MDA was significantly ($p < 0.05$) increased compared to control, while the level of GSH was reduced dramatically ($p < 0.05$). Both observations were detected in kidney and liver tissues. Interestingly, GB-EE treatment along with $H_2O_2$ exposure significantly ($p < 0.05$) improved the abnormal changes in the level of MDA and GSH compared with mice group treated with H2O2 only. GB-EE treatment resulted in reduction of MDA level by 53.8 and 52.2% in kidney and liver tissue, respectively after 9 days exposure to $H_2O_2$. On the other hand, the extract resulted in 2.28- and 3.1-fold increase in the level of GSH in kidney and liver tissue, respectively.

**(Table 3): Mean ± SD MDA and GSH level in mice liver and kidney tissues upon different treatments.**

| Groups | MDA (Mean ± SD) nmol/g | |
|---|---|---|
| | Kidney | Liver |
| **I** | 0.31±0.02a | 0.33±0.04a |
| **II** | 0.36±0.04a | 0.35±0.04a |

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| | | |
|---|---|---|
| **III** | 1.19±0.12b | 1.34±0.14b |
| **IV** | 0.64±0.07c | 0.7±0.08c |
| **Groups** | **GSH (Mean ± SD) mg/g** | |
| | **Kidney** | **Liver** |
| **I** | 0.86±0.06a | 0.90±0.08a |
| **II** | 0.79±0.08a | 0.84±0.10a |
| **III** | 0.25±0.03b | 0.22±0.02b |
| **IV** | 0.57±0.04c | 0.68±0.05c |

**Different letters (a, b, c) considered significantly different at $p < 0.05$.**

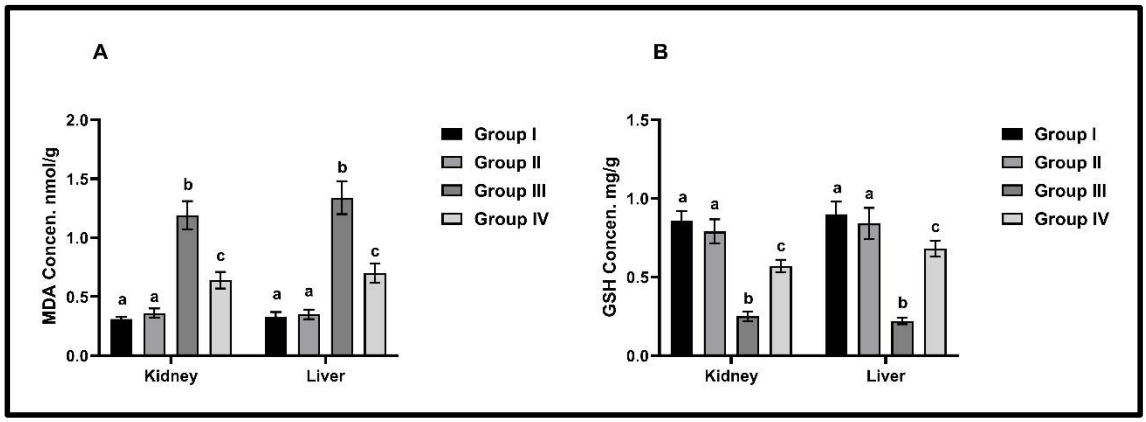


**Fig. 1: Effects of GB-EE treatment on MDA and GSH levels of mice liver and kidney tissues treated with $H_2O_2$. Different letters (a, b, c) are significantly different at $p < 0.05$.**

This study demonstrates that GB-EE treatment exhibits a protective effect against biochemical toxicity induced by $H_2O_2$. The protective function of GB-EE is linked to various mechanisms, notably the activation of antioxidant enzymes [26]. Ginkgolide, a component of *G. biloba* extract, has been shown to protect cells from necrosis and apoptosis by modulating glutathione peroxidase activity and reducing malondialdehyde (MDA) levels associated with lipid peroxidation [27]. Consequently, GB-EE safeguards liver and kidney tissues from damage caused by $H_2O_2$, decreases MDA levels, and restores the compromised antioxidant balance. A significant increase in MDA levels and a decrease in GSH levels were observed in the $H_2O_2$ -treated group. Elevated concentrations of $H_2O_2$ may induce oxidative stress, leading to cellular damage [28]. The antioxidant systems within cells protect against oxidative damage. The elevation of oxidant levels and inadequate antioxidant capacity result in irreversible cellular damage. GSH serves as a crucial antioxidant within cells, and a reduction in GSH levels signifies oxidative damage [29]. MDA results from lipid peroxidation, and elevated levels of MDA indicate oxidative damage within the cell [30]. Administration of GB-EE resulted in partial enhancement of GSH and MDA levels, demonstrating a distinct protective effect against oxidative damage.

## Conclusions

The *G. biloba* ethanolic extract (GB-EE) exhibited significant antioxidant activities, including potent free radical scavenging and moderate reducing power. The extract's rich flavonoid content contributed to these antioxidant properties. Furthermore, GB-EE demonstrated a protective effect against

oxidative stress-induced toxicity *in vivo*, highlighting its therapeutic potential for conditions characterized by redox imbalance

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## Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

## Author's Contribution Statement

[First Author]: Contributed to the conception and design of the study, conducted all experiments, data rearrangement and drafted the initial manuscript.

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