



Research Article

Evaluation of General Toxicity, Anti-Oxidant Activity and Effects of *Ficus Carica* Leaves Extract on Ischemia/Reperfusion Injuries in Isolated Heart of Rat

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Abstract

Purpose: This study was aimed to evaluate general toxicity, anti-oxidant activity and effects of *Ficus carica* leaves extract on ischemia/reperfusion injuries.

Methods: Antioxidant activity, total phenolic and flavonoid compounds of 70% methanolic extract of *Ficus carica* leaves were measured. The general toxicity test was carried out by brine shrimp lethality assay. Isolated hearts of male rats were mounted on a Langendorff apparatus and perfused with modified Krebs-Henseleit solution. In control group, the hearts were perfused with normal Krebs solution, however, treatment groups received enriched solution with the extract (0.04, 0.2 and 1 mg/ml) during stabilization and reperfusion (after 30 min global ischemia), respectively. Cardiac arrhythmias were analyzed and TTC method was used for infarct size determination.

Results: The extract displayed antioxidant activity in the DPPH assay ($RC_{50}=0.06666$ mg/ml). Total phenolic content was 12.29 mg GAE/100 g dry sample and the amount of flavonoids was calculated 40.729 mg/g. LD50 value by brine shrimp test was 0.158 mg/ml. The extract decreased number of VEBs, incidence and duration of Rev VF with clear reduction in infarct size and infarct volume ($P<0.001$).

Conclusion: *Ficus carica* decreased ischemia/reperfusion-induced injuries. These protections are probably due to antioxidant capacity and the existence of flavonoid and phenolic compounds in the extract.

Introduction

According to American Heart Association, most of deaths in developed countries are due to cardiovascular diseases (CVD).¹ Atherosclerosis and inflammation play key roles in heart diseases² and smoking, hypertension, dyslipidemia, diabetes and sedentary lifestyle are major risk factors for prevalence of CVD.³

Free radicals are generated in physiological processes, but under normal condition, a variety of antioxidant mechanisms can control them; however the increased production of them will cause lipid peroxidation⁴ that could increase the risk of most common diseases, particularly coronary heart disease and cancer.^{2,5}

The main biomolecules which are responsible for a significant role in preventing the diseases are phenolic compounds. These compounds play an important physiological role in plants and are also necessary for human health. The antioxidant activity of them can result through different ways: as reducing agents, hydrogen donors, recipients of free radical rejection of the single oxygen and generally act as the savior of the cells.⁶ Phenolic compounds in plants are one of the key ingredients that have widespread therapeutic uses. Since

these compounds can modulate lipid peroxidation, thereby they can be used in atherogenesis, thrombosis and carcinogenesis.⁷ Certain plants have been used for thousands of years in traditional medicine.^{5,8} Fig plant is one of the only five plants mentioned in the holy Quran, along with olives, grapes, pomegranate and dates. Its fruit, root and leaves are used in the traditional medicine for different diseases such as metabolic, respiratory, antispasmodic and anti-inflammatory disorders for centuries.^{9,10} It was found that the leaves of Fig [*Ficus carica* Linn. (Syn: *Ficus sycomorus*; family: Moraceae)] have high total phenol and flavonoid content which can act as powerful antioxidants.¹⁰ *Ficus carica* leaves are also used in cancer and dermatitis.¹¹ It has stronger antioxidant properties than the pulp. Some researchers also suggested that there is a higher amount of phenolic substances in the leaves of the plant in comparison with stem bark and fruit.¹¹

Since no study has been done about the potential protective properties of *Ficus carica* leaves extract in the heart in ischemic conditions. Hence, the aim of this study was to evaluate general toxicity, anti-oxidant activity and

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its effect on ischemia/reperfusion (I/R) induced cardiac arrhythmias and infarct size in isolated heart of rat.

Materials and Methods

Plant material and extraction method

Leaves of *Ficus carica* were collected during August and September, 2013, from Azarshahr city (northwest of Iran) and the identity was confirmed by anatomical examination in comparison with the herbarium specimen retained in the School of Pharmacy, Tabriz University of Medical Sciences, Iran. Dried and ground *Ficus. carica* leaves (100g) were macerated using 70% methanol. Then the methanol of the extract was evaporated under vacuum at 50°C by rotary evaporator to get a dried extract; then stored in refrigerator (2-8°C) for further analysis.

DPPH radical scavenging assay

2, 2- diphenyl -1- picrylhydrazyl (DPPH) assay most commonly involves a hydrogen atom transfer. A solution of DPPH (0.08 mg/ml) in methanol was used. The compound was dissolved in methanol to obtain a concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 1.56×10^{-2} , 3.13×10^{-2} , 6.25×10^{-2} , 12.5×10^{-2} , 25×10^{-2} mg/ml. Diluted solutions (5 ml each) were mixed with DPPH (5 ml) and allowed 30 min for any reaction to occur at room temperature. The UV absorbance was recorded at 517 nm. The experiment was performed in duplicate and average absorption was noted for each concentration. The data were processed using Excel and the concentration that caused a 50% reduction in absorbance (RC50) was calculated.¹²

Total phenol content

Total phenol content was measured according to Heshmati Afshar et al. (2012). One ml of each sample (5 mg in acetone: water (60:40) v/v) was added to 0.2 ml Folin-ciocalteau's reagent (1:2 diluted water) and 1 ml of 2% Na₂CO₃ mixture. After 30 min incubation at room temperature, their absorbance was measured at 750 nm. For the calibration curve, 10 mg of Gallic acid was dissolved in 10 ml of acetone: water (60:40) v/v as a stock solution. Experiments were repeated 2 times for every dilution and calibration curve was created.¹³

Total flavonoid content

Total flavonoids in of 70% methanolic extract of *Ficus carica* leaves were determined by AlCl₃ method. Sample solutions were prepared in 80% methanol. To prepare AlCl₃ reagent, 133 mg crystalline aluminum chloride and 400 mg crystalline sodium acetate were dissolved in 100 ml of 80% methanol. For flavonoid estimation, to 2 ml of sample, 400 µl of water and 1 ml of AlCl₃ reagent were added. Absorbance was recorded at 430 nm against the blank. Quercetin (1 mg/ml) in 80% methanol was used as a stock solution. The amount of flavonoids was calculated in quercetin equivalent from the calibration curve of quercetin (5-25 µg/ml).¹⁴

General Toxicity

Brine Shrimp lethality assay was carried out to investigate the toxicity of *Ficus carica* leaves total extract. Artificial sea water was prepared with commercial salt for hatching brine shrimp eggs (for 24 hours: 25-29 °C). After 48 h, 10 brine shrimps were transferred to each sample vial containing 4.5 ml of brine solution. A 0.5 ml of plant extract which dissolved in DMSO was added to 4.5 ml of brine solution. After performing dilution series, containers were maintained for 24 h and the surviving larvae were counted for determination LD₅₀.^{15,16} In cases where control deaths occurred, the data were corrected using Abbott's formula: [(test-control)/ control]×100.¹⁷

Animals

In this study, male Wistar rats weighing 250-300 g were used. The animals were provided with suitable environmental and nutritional conditions. This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences (National Institutes of Health Publication No 85-23, revised 1985).

Isolated heart perfusion

The animals were randomly divided into four groups (8-10 rats in each). Their hearts were rapidly excised after anaesthetizing by 60 mg/kg sodium pentobarbital and heparin sodium (300 IU/rat) intraperitoneally then mounted via the aorta on Langendorff perfusion apparatus and perfused with modified Krebs-Henseleit bicarbonate buffer contains (in mM): NaCl 118.5, NaHCO₃ 25.0, KCl 4.8, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.7 and D-glucose 12.0. All solutions were gassed with 95% O₂/5% CO₂ with pH maintained between 7.35-7.45 at 37°C. A latex, fluid filled, isovolumic balloon was introduced into the left ventricle through the left atrial appendage and connected to a pressure transducer (P-1000B; Narco Bio Instruments). The ECGs were recorded by Powerlab system for analysing cardiac arrhythmias including ventricular ectopic beats (VEBs), ventricular tachycardia (VT) and ventricular fibrillation (VF). After 20 min stabilizing with Krebs-Henselit buffer in all groups, the hearts were subjected to 30 min global ischemia followed by 120 min reperfusion. In control group, the hearts were perfused with Krebs-Henselit solution throughout the experiment, however, treatment groups received enriched Krebs solution with total *Ficus carica* leaves extract (0.04, 0.2 and 1 mg/ml) during 20 min stabilization and 120 min reperfusion (after 30 min global ischemia), respectively.^{18,19}

Infarct size determination

After ending 120 min reperfusion, the hearts were cut into slices and incubated by 1% Triphenyltetrazolium chloride (TTC) solution (15 min in 37°C) and fixed by formalin. The area of at risk and infarct size were determined by computerized planimetry.²⁰

Statistical analysis

Except for the incidence of VT and VF which are expressed as percentage, all the other results are expressed as Mean \pm SEM. To compare the number of VEBs, VT and duration of VT and VF between groups, the Mann-Whitney non-parametric U-test was employed. For analyzing the incidence of VT and VF, Fisher exact test (Chi-square with Yates correction) was used. The mean percentage of infarct size was analyzed using one-way ANOVA and then significant differences were examined by LSD post hoc range test. Differences between groups were considered significant at a level of $P<0.05$.

Results

Free Radical-Scavenging Activity of *Ficus carica* Leaves

In this study, DPPH method was used for evaluating free radical scavenging activity of *Ficus carica* leaves. Antioxidants are substances which reduce the radical form of DPPH through a donation of electron or hydrogen; by this reaction, DPPH changes the color from purple to yellow. The scavenging activity of *Ficus carica* leaves was measured by RC_{50} . The lower RC_{50} indicates the more potent antioxidant capacity. Compared to standard antioxidant Quercetin (RC_{50} : 0.0039 mg/ml), the RC_{50} of 70% methanolic extract of *Ficus carica* leaves was 0.06666 mg/ml.

Total Phenolic Content

Total phenolic content was determined in comparison with standard Gallic acid. It was found that the total

phenolic content of this extract is 12.29 mg GAE/100 g dry extract.

Total flavonoids Content

Flavonoids are the main class of polyphenols which have numerous pharmacological effects.²¹ This class has been determined by Aluminum Chloride method¹⁴ and the amount of flavonoids was calculated by quercetin standard curve (40.729 mg/g).

Brine Shrimp Lethality Bioassay

Toxicity of 70% methanolic extract of *Ficus carica* leaves was detected by Artemia Salina bioassay. The LD₅₀ value of brine shrimp obtained from the extract was 0.158 mg/ml.¹⁵

Effects of 70% methanolic extract of *Ficus carica* leaves on ischemia/reperfusion induced arrhythmias

The effect of total extract of *Ficus carica* leaves on cardiac arrhythmia are shown in Table 1. In comparison with the control group, administration of the extract reduced number of some reperfusion-induced arrhythmias such as Single, Salvos and total VEBs especially by higher concentrations. As shown in Table 1, the number of total VEBs was 40 \pm 11 (control), however, 1 mg/ml of the extract decreased it to zero ($P<0.001$). At the same time, incidence and duration of Re VF were significantly lowered by the above concentration ($P<0.001$ for both of them). On the other hand, 0.2 and 1 mg/ml of the extract increased incidence of Irre VF compared to the control group (Table 1).

Table 1. The effects of 0.04-1 mg/ml of methanolic extract of *Ficus carica* leaves on the first 30 min reperfusion arrhythmias after 30 min global ischemia in isolated heart of rat.

| Arrhythmias | | Control | Extract (0.04 mg/ml) | Extract (0.2 mg/ml) | Extract (1 mg/ml) |
|----------------|----------|-------------|-------------------------|------------------------|----------------------|
| Count | Single | 33 \pm 9 | 10 \pm 3 | 8 \pm 7** | 0** |
| | Salvos | 8 \pm 3 | 2 \pm 1 | 2 \pm 1 | 0** |
| | VT | 0 | 0 | 0 | 0 |
| Total VEBs | | 40 \pm 11 | 11 \pm 4 | 10 \pm 9** | 0*** |
| Duration (sec) | VF | 0 | 0 | 0 | 0 |
| | Re VF | 54 \pm 8 | 15 \pm 7** | 175 \pm 98 | 0*** |
| Incidence (%) | Re VF | 100 | 100 | 44* | 0*** |
| | Irre VF | 0 | 0 | 56* | 100*** |
| | Total VF | 100 | 100 | 100 | 100 |

VT: ventricular tachycardia, Re VF: Reversible Ventricular Fibrillation, Irre VF: Irreversible Ventricular Fibrillation. Data are expressed as mean \pm SEM. *** $P<0.001$, ** $P<0.01$, * $P<0.05$ versus control.

Effects of total extract of *Ficus carica* leaves on ischemia/reperfusion induced myocardial infarction

The results showed that perfusion of 70% methanolic extract of *Ficus carica* leaves produced significant reduction in myocardial infarction size. The value was 56.6 \pm 4.9% in the control group (Figure 1), however

administration of the extract (0.04, 0.2 and 1 mg/ml) reduced infarct size to 12.5 \pm 4.5, 12.6 \pm 3.3 and 15.7 \pm 3.1 % ($P<0.001$ for all treated groups), respectively. There was no statistical difference between the effects of various concentrations of the extract in treated groups. Similar to the infarct size results, administration of the

extract significantly decreased the infarcted volume of ischemic hearts in comparison with the control group by all used concentrations (Figure 2).

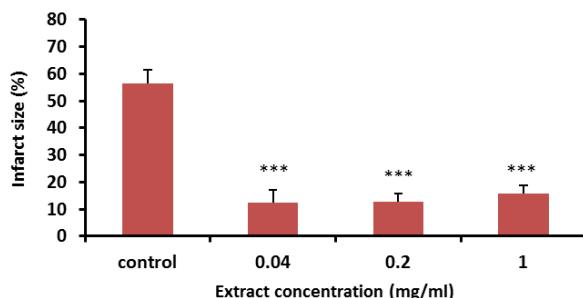


Figure 1. Percentage of infarct size in the control and isolated rat hearts receiving 0.04-1 mg/ml of 70% methanolic extract of *Ficus carica* leaves during 120 min reperfusion after 30 min global ischemia. Data are expressed as mean \pm SEM. *** P<0.001 in comparison with the control group.

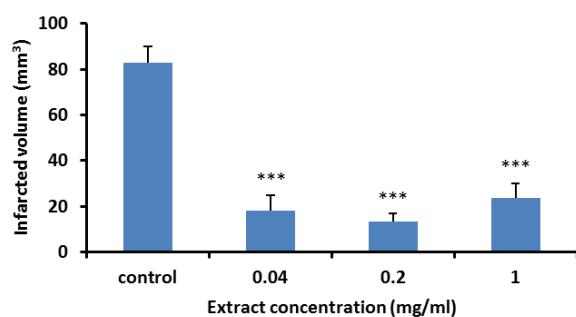


Figure 2. The volume of infarcted area in the control and isolated rat hearts receiving 0.04-1 mg/ml of 70% methanolic extract of *Ficus carica* leaves during 120 min reperfusion after 30 min global ischemia. Data are expressed as mean \pm SEM. *** P<0.001 in comparison with the control group.

Discussion

Medicinal plants are the world's heritage, which play an important role in rural people's lives, particularly for who live in places with inaccessible health facilities. In the present study, we tried to bring *Ficus carica* as a traditional medicine into a medical laboratory for recognizing the potential protective effects of it on ischemic heart. For the first time, we evaluated general toxicity, anti-oxidant activity and effect of *Ficus carica* leaves extract on ischemia/reperfusion induced cardiac arrhythmias and infarct size in isolated heart of rat. The results showed that 70% methanolic extract of *Ficus carica* leaves had significant effect on the reduction of infarct size. Compared to the control value, the extract (0.04, 0.2 and 1 mg/ml) lowered infarct size to 77.9, 77.7 and 72.2 % (P<0.001 for all), respectively. In addition, the infarcted volume was significantly decreased by the extract. Moreover, administration of the extract reduced number of VEBs, incidence and duration of Re VF with increasing in the incidence of Irre VF (Table 1).

Regarding the existence of important flavonoids and phenolic compounds in the extract, it seems that some

antiarrhythmic effects and reduction of infarct size and infarct volume may relate to the extract's antioxidants. As it was stated in the results section, the extract had considerable amount of flavonoid and phenolic compounds which have the ability to reduce free radical formation and scavenge them^{11,22}. This important relationship between flavonoids, phenolic compounds and antioxidant activity has been proven in past years²³⁻²⁵ and our data confirms it again where total phenolic and flavonoid content of the extract was 12.29% mg GAE/100 g and 40.729 mg/g dry 70% methanolic extract, respectively. RC50 of it through DPPH method was 0.06666 mg/ml.

Free oxygen radicals may be involved in many cardiovascular diseases via oxidation of LDL, which play an important role in atherosclerosis.^{25,26} Recent studies confirm that some species of *Ficus* have protective activities in oxidative damage, diseases such as inflammation, cancer and diabetes.²⁷⁻³¹ This plant's fruit, root and leaves are also used in the treatment of gastrointestinal (especially constipation), respiratory, inflammation and cardiovascular disorders^{27,32}. As well as anti-inflammatory effect, the ripe dried fruit of *Ficus carica* has anti-spasmodic and anti-platelet properties. The total extract of *Ficus carica* was evaluated for hepatoprotective activity in CCl₄-induced liver damaged rats.³³ Different research groups have demonstrated that *Ficus carica* aqueous stem extract could reduce methanol-induced toxicity, particularly hepatotoxicity, by suppressing alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase (LDH) activities, inhibiting lipid peroxidation and increasing antioxidant enzyme activities.³⁴ LDH is an isoenzyme which increases through tissue damages. This biomarker has been used for many years to assess ischemia induced injuries and diagnosis and prognosis of acute myocardial infarction.^{35,36} Maybe another mechanism of the extract for lowering I/R-induced injuries is suppression of LDH. In addition, this worthy plant has been recommended one of the herbal sources of future drugs and food supplements because of its effective property in oxidative related diseases.³⁷

Conclusion

Results of this study showed that administration of 70% methanolic extract of *Ficus carica* leaves produces protective effects against I/R-induced myocardial infarction in isolated rat heart. The mechanisms for this protection are probably due to antioxidant capacity and the existence of flavonoid and phenolic compounds in the extract. Further investigations are needed to identify the exact mechanism of action of the extract and its effective ingredients.

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Conflict of Interest

There is no conflict of interest to be reported.

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