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Research Article

Protective Role of *Ficus carica* Extract Against Hepato-Testicular Side Effects and Genotoxicity Induced by Cisplatin

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ABSTRACT

Objective: The present work investigated the protective effect of *Ficus carica* (common fig) leaves methanol extract against genotoxicity and testicular damage of cisplatin (CP) and identified some of its active ingredients. **Methods:** Seven main groups were investigated as follows: I. control negative, II. Control plant (600 mg/kg fig, orally), III, IV. Control positive (treated i.p with 10 and 15 mg/kg CP), V-VII. groups treated with fig (200, 400 and 600 mg/kg) + Cisplatin (15 mg/kg). **Results:** *Ficus carica* alleviated the destructive effects of CP in the testis, liver and bone marrow due to the presence of high amount of flavonoids and phenolic compounds. Also it has a normal effect in the tested parameters as compared with the control negative. Chromatographic investigation resulted in the identification of 6 compounds: Catechin, Luteolin-8-C-β-D glucopyranoside, Quercetin, Quercetin-3-O-β-d-glucopyranoside, Chlorogenic acid and Kaempferol-3-O-β-D-glucopyranoside. In bone marrow cisplatin induced significant percentage of chromosome abnormalities, micronuclei in polychromatic erythrocytes and toxicity to cells. On the contrary the two tested doses of cisplatin had a normal effect on spermatocyte chromosomes (germ cells). The dose 15 mg/kg induced an overexpression of the liver genes NF-kB and iNOS as indicated by real-time PCR. Different forms of histopathological alterations and instigation of the expression of TNF-α gene in the testis were detected after CP treatment. **Conclusion:** *Ficus carica* is a promising candidate rich in many bioactive constituents and can be used in combination with chemotherapeutic drugs to alleviate their destructive effects.

Key words: Cisplatin, Bone marrow, Spermatocytes, liver, Testis, Fig, Protection.

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INTRODUCTION

Plant products and their secondary metabolites are generally safer and cheaper compared to synthetic drugs and could be used for the treatment of many diseases. Herbal medicine is considered nowadays the point of interest for many pharmacologists who try to produce new drugs from plant products[1].

Ficus carica Linn (Moraceae) is an important plant in the genus Ficus and commonly referred to as "fig". It is one of the first plants that were cultivated in the world. *Ficus carica* is an important genetic resource with high nutritional and economic values. It has been used in traditional medicine for the treatment of a wide range of ailments related to digestive, endocrine, reproductive and respiratory systems. It is also used in the gastrointestinal tract, urinary tract infections and for inflammatory-related diseases and cancer. Phytochemical studies on fruits and leaves of fig have shown that they are rich in phenolics, organic acid and volatile compounds[2]. Extracts from different parts were demonstrated to possess a wide range of biological activities. Some of the most interesting therapeutic activities include: immunosuppressive[3], anti-inflammatory[4], hepatoprotective[5] , hypoglycemic[6] and anticancer activities[7].

In the present work cisplatin is used as a positive genotoxic agent. Cisplatin (CP) is one of the first-line anticancer drugs that has gained widespread use against various forms of human malignancies *e.g* testicular, ovarian and breast cancers, esophageal cancer, lung cancer, bladder cancer, head and neck cancer, brain tumors and neuroblastoma[8]. Cisplatin is from the platinum-based antineoplastic family of medications. It works in part by binding to DNA and inhibiting its replication[9]. Like other chemotherapeutic drugs, cisplatin possesses many adverse side effects in patients under its treatment including: genotoxicity[10], nephrotoxicity[8], liver dysfunction[11] and reproductive impairment[12].

The present work aimed to evaluate the possible protective effect of *Ficus carica* leaves methanol extract against genotoxicity and testicular damage of cisplatin. The study includes genetic and histopathological endpoints. Phytochemical studies were performed to separate and identify some of the active components of *Ficus carica* and their relation with the obtained results was discussed.

MATERIALS AND METHODS

First part: Phytochemical studies

Preparation and chemical investigation of Ficus carica extract

The leaves of *Ficus carica* were collected from Giza governorate, Egypt, in Sep. 2018, and identified by

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Dr. Adel Salama. A voucher specimen is deposited at the National Research Centre herbarium, under the number of F 232. The plant leaves were dried at room temperature and finely powdered.

General experimental conditions

Column chromatography was performed on sephadex LH-20 (Fluka) and polyamide 6S (Riedel-De Haen, Hannover, Germany). Paper chromatography Whatman No. 1 and 3 MM were carried out using solvent systems: A (15% AcOH) and B (n-BuOH-AcOH-H2O, 4:2:1). Mass spectra were achieved on Finnigan SS Q 700 spectrometer, 70 eV. NMR experiments were performed on a Bruker AMX 400 instrument with standard pulse sequences operating at 400 MHz in ^{1}H NMR and 100 MHz in ^{13}C NMR. Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard and DMSO-d_6 as solvent at room temperature. UV spectral data was measured on a Shimadzu 240 spectrometer in MeOH.

Extraction

Dried leaves powder of *Ficus carica* (1 kg) was extracted with MeOH four times in a percolator. The collected MeOH extracts were evaporated under vacuum (40 ºC) to yield 152.55 g. The MeOH extract was kept under cooling (10 ºC) until analysis.

Determination of total phenolics and flavonoids

Reagents: Aluminum chloride was prepared by dissolving 2 g of aluminum chloride (Sigma) in 100 ml pure methanol (HPLC). Foline reagent, saturated solution of sodium carbonate (40 g /100 ml distilled water).

The determination of the total phenolic and flavonoids in the methanolic extract of *Ficus carica* leaves was carried out as follows, a known weight of MeOH extract was dissolved in 80% methanol, transferred to measuring flask 100 ml and completed to 100 ml with 80% methanol.

Determination of total phenolic

The total phenolic content was determined by Folin–Ciocalteu according to the method described by Meda *et al.*[13] with some modifications. One ml of the sample extract was added to 7.5 ml of redistilled water and 0.5 ml of Folin–Ciocalteu reagent (Sigma Chemical Co.; St. Louis, Mo.; U.S.A.) was added. After 15 min equilibration, the mixture was neutralized with 1.0 ml of Na_2CO_3 40%, mixed well by a vortex. After a 30 min reaction, the absorbance of the mixture was measured at 760 nm. To methanol 80%, (8.5 ml), 0.5 ml Folin and 1 ml Na_2CO_3 was used as blank. Chlorogenic acid (Sigma) was used as a standard curve. Concentration of the total phenolic was determined from a standard calibration curve. The mean of three readings was used and results were expressed as milligrams of chlorogenic acid equivalent per gram of the extract (mg/g extract).

Determination of total flavonoids

The total flavonoid content was determined according to the method adopted by Meda *et al.*[13] (2005). Briefly, 3 ml of the extract were mixed with 3 ml of $AlCl_3$ (2 % in methanol) and the mixture was allowed to stand for 30 min. The absorbance was measured at 415 nm against a blank sample which consists of 3 ml $AlCl_3$ with 3 ml methanol 80 %. Rutin (Sigma–Aldrich) was used as a standard and the total flavonoid content was determined using the standard curve, the mean of three readings was recorded and the total flavonoids were expressed in mg as rutin equivalent per 1g of the extract (mg /1g extract).

Isolation of the phenolic constituents

The methanol extract was suspended into hot water, left overnight, filtered and was successively partitioned with methylene chloride and *n*-butanol (BuOH) and then evaporated till dryness under vacuum (53 and 38 g, respectively). The BuOH fraction was subjected to TLC and paper chromatography in two different systems; a (15% AcOH) and B (*n*-BuOH-AcOH-H_2O). The dry BuOH extract was uploaded on a polyamide 6S column chromatography (100 x 5 cm). The column was eluted with H_2O, and then H_2O-MeOH mixtures of decreasing polarity and seven fractions (1 L, each) were collected. The major phenolic fractions obtained were combined into two fractions after chromatographic analysis. Fraction A (1.0 g) was fractionated by column chromatography on sephadex LH-20 with aqueous MeOH (0-80 %) for elution to give compounds 1 (17 mg), 2 (13 mg) and 3 (33 mg). Fraction B (1.5 g) was chromatographed on sephadex LH-20 column and MeOH (90%) to yield pure compounds 4 (27 mg), 5 (19 mg) and 6 (22 mg).

Second part: Biological studies

Chemicals

Cisplatin (Cis-Diamminedichloroplatinum (II) Pt $(NH_3)_2Cl_2$ was purchased from *Alfa Aesar* (PRODUCT OF UNITED STATES), CAS: 15663-27-1. EINECS: 239-733-8.

Experimental animals

Mature male Swiss Albino mice *(Mus musculus*), weighing about 20-25 grams were obtained from the animal house colony of the National Research Centre (Dokki, Cairo, Egypt). The animals were housed in stainless steel wire mesh cages on a bedding of wood chips, kept in an ambient temperature of 25±3ºC on a light/dark cycle of 12/12h and supplied with food and water *ad-libitum*.

Ethical consideration

This prospective study was reviewed and approved by the Animal Ethics Committee of the National Research Centre, Cairo, Egypt (approval number: 1.6.2.1.0) and was carried out according to the National Institute of Health Guide (NIH) for the care and use of laboratory animal's guidelines.

Experimental design and doses

After one week of acclimatization, 70 mice were fasted overnight before treatment and were randomized into 7 equal groups (10 mice/ group) as follows**:**

***I. Control negative**, **II. Control plant** (treated orally for 5 consecutive days with 600 mg/kg fig), **III, IV. Control positive** (treated i.p with 10 and 15 mg CP, a single dose treatment for 24 h), **VI-VII. Fig + cisplatin:** Three groups treated with Ficus carica (200, 400 and 600 mg/kg, orally, 5 consecutive days) + Cisplatin (injected once at the last day of treatment 24 h after sacrificing)*

In all experiments animals were anesthetized using diethyl ether and then killed by cervical dislocation 24 h after the last treatment. Mice in each group were subdivided into two sub-groups (5 animals/each), one for micronucleus and histopathological studies (without colchicine). The second subgroup was injected i.p with colchicine (40 mg/kg) 2.5 h before sacrificing and was subjected to chromosomal aberration analysis. Cisplatin and *Ficus carica* extract were carefully dissolved in distilled water.

Experimental Procedures

Genotoxic study

Micronucleus test

The micronucleus preparation from bone marrow of treated mice was performed following the standard test protocol and according to the Guideline (OECD) 474 for Testing of Chemicals[14]. Both femurs were removed and bone marrow flushed with Fetal Bovine Serum (FBS) about 3 mL. Cells were centrifuged at 2000 rpm for 5 min and smeared on slides. The air-dried slides were fixed by submerging in absolute methanol (for 10 ~ 20 min). Fixed slides were stained with May-Grunwald and Giemsa stains. Micronuclei were identified as dark blue staining bodies in the cytoplasm of polychromatic erythrocytes (PE's). The ratio of erythrocytes to nucleated cells was determined and expressed as the percentage of PE's/100 nucleated cells (PE's + NE's). 2000 nucleated cells were counted per each animal (5 animals/group). The cells were scored under a light microscope at a 1000× magnification.

Chromosomal aberration assay in bone marrow and mouse spermatocytes

Bone marrow chromosomes were prepared according to the technique described by Fahmy *et al.*[1] In brief, mouse bone- marrow cells were collected from both femurs, cells were incubated in hypotonic solution (KCL 0.075 M) for 20 min at 37°C, and then centrifuged. The cell pellets were suspended in a fixative (methanol/glacial acetic acid 3:1). This step was repeated at least twice, then the cells suspended in a few drops of fixative and spread onto frozen slides, air dried, stained with 10% Giemsa for 30 min, washed, and air dried again.

Spermatocyte chromosomes were prepared from the testes of the same animals according to the protocol described by Hassan *et al.*[15] . Briefly, the testis was removed and squashed into a petri dish containing an isotonic solution 2.2% trisodium citrate. Then the cell suspension was centrifuged for 5 minutes at 1500 rpm. The cell pellet was incubated in a hypotonic solution 1.1% trisodium citrate for 20 minutes at 37C° followed by centrifugation. The cell pellet was washed twice by a freshly prepared fixative. A few drops of the fixative cell suspension were dropped in a clean microscopic slide, air dried and stained with 10% Giemsa stain.

In each a hundred well spread metaphases were analyzed per mouse describing different kinds of abnormalities. Scoring was performed under 2500× magnification with a light microscope.

Quantitative Real Time-PCR (qRT-PCR) for analysis of hepatic nuclear factor-kappa B (NF-kB), inducible nitric oxide synthase (iNOS) mRNA expression:

Total RNA was isolated from 500 µl of liver samples of mice by the standard TRIzol extraction method (Invitrogen, Paisley, UK). To remove any possible genomic DNA contamination, the total RNA samples were pre-treated using DNA-free™ DNase treatment and removal of reagents kit (Ambion, Austin, TX, USA) following the manufacturer's protocol and were recovered in 100 µl molecular biology grade water and stored at -20°C. The RNA concentration and purity were determined by Nanodrop Spectrophotometer absorption (Thermo Scientific, USA) at 260 nm[16].

Complementary DNA (cDNA) was generated using Superscript Choice Systems (Life Technologies, Breda, Netherlands) according to the manufacturer's instructions. To assess the mRNA expression of NF-kB, iNOS, quantitative real-time PCR was performed using SYBR green PCR Master Mix (Applied Biosystems, CA, USA) as described by the manufacturer. Briefly, in a 25 µl reaction volume, 5 µl of cDNA were added to 12.5 µl of 2× SYBR green Master Mix and 200 ng of each primer. The sequences of primers are described in Table 1. The temperature profile was as follows: 94 ∘C for 3 min, 94∘C for 20 s, 60 ∘C for 20 s and 72∘C for 20 s for 35 cycles. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of (GAPDH) as housekeeping gene by the ΔΔCt method[17].

Histopathological studies

Specimens of testes were dissected and fixed in 10% buffered formalin-saline for 72 hours, washed in tap water for half an hour, dehydrated in ascending grades of alcohol, cleared in xylene, impregnated in soft paraffin wax at 55ºC and embedded in hard paraffin. Serial sections of 5-6µm thick will be cut and stained with H&E stain and analyzed by a light microscope at 400× magnification[18]. Images will be captured and processed using Adobe Photoshop Version 8.

Immunohistochemical assessment of TNF-α expression

Preparation was carried according to the technique described by Jammal *et al.*[19]. Immuno-histochemical staining of tumor necrosis factor-alpha (TNF-α) antibody was performed with sections of 4 µm thick that were deparaffinized and incubated with fresh 0.3 % hydrogen peroxide in methanol for 30 min at room temperature. Deparaffinized testis slides were incubated with the antibodies against caspase-3 diluted 1:100, and positive cells were determined with streptavidin-biotin-peroxidase secondary antibody (DAKO Universal). The binding sites of antibody were visualized with DAB. The sections were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted. The immunostaining intensity and cellular localization of TNF-α were analyzed by light microscope.

Statistical analysis

Data were computerized and analyzed using Statistical Package of Social Science (SPSS Inc., version 20, Armonk, New York: IBM Corp). One way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was used to determine the difference among the means. The level of statistical significance was set at $P < 0.05$.

RESULTS

First part: phytochemical studies

Total phenolic and flavonoid contents of *Ficus carica*

The phenolic content of *Ficus carica* was determined in MeOH extract using Folin- Ciocalteu reagent. While, the total flavonoid content was determined using $AlCl_3$ reagent. The results showed that the MeOH extract of this plant contains a high content of total phenolics which reached 63.37 mg/g extract and total flavonoids (59.67 mg/g extract). The total phenolic and total flavanoid contents in methanol extract were expressed as chemical equivalents of chlorogenic acid and rutin, respectively.

Table 1: represents the sequence of forward and reverse primers.

Gene		Primer sequence (5′-3′)	Primer size (bp)
NFk-B	F	CATGAAGAGAAGACACTGACCATGGAAA	329
	R	TGGATAGAGGCTAAGTGT AGACACG	
iNOS	F	TGTGTCAGCCCTCAGAGTAC	312
	R	CACTGACACTYCGCACAA	
GAPDH	F	cggctactagcggtttta cg	188
	R	aagaagatgcggctg act gt	

Identification of isolated phenolic compounds:

› Compound 1 (Chlorogenic acid):

This compound showed chromatographic properties and color reactions similar to those reported for "chlorogenic acid (blue spot on PC under UV light, turning bright yellow when fumed with ammonia vapor, changing to yellow with $AlC1_3$ spray reagent [26]. Mass spectrum showed a molecular ion (M+) at m/z 354, which corresponds to the molecular formula $C_{16}H_{17}O_9$. The ^{1}HNMR spectrum (400 MHz, DMSO- d_6) showed signals, 6.34 (H-8`, d, J=16.0), 6.84 (H-5`, d, J= 8.4), 7.03 (H-6`, dd, J = 8.5, 1.8), 7.13 (H-2', d, J= 1.8), 7.58 (H-7`, d, J=16.0). From the above chromatographic and spectroscopic data, compound 1 could be identified as chlorogenic acid"[20].

› Compound 2 (Catechin):

^{1}H NMR (400 MHz, DMSO-d_6): δ "6.84 (1H, d, *J* = 2.0 Hz, H-2'), 6.77 (1H, dd, *J* = 8.1 Hz, H-5'), 6.71 (1H, dd, *J* = 2.0, 8.1 Hz, H-6'), 5.92 (1H, d, *J* = 2.4 Hz, H-8), 5.85 (1H, d, *J* = 2.4 Hz, H- 6), 4.56 (1H, d, *J* = 8.0 Hz, H-2), 3.97 (1H, ddd, *J* = 8.0, 8.0, 4.8 Hz, H-3), 2.85 (1H, dd, *J* = 4.8, 16.0 Hz, H-4), 2.50 (1H, dd, *J* = 8.0, 16.0 Hz, H-4)". ^{13}C NMR (100 MHz, DMSO-d_6): δ "27.7 (C-4), 66.3 (C-3), 80.9 (C-2), 93.9 (C-6), 95.1 (C-8), 114.5 (C-2'), 115.1 (C-5'), 18.4 (C-6') and other aromatic carbons showed peaks at *δ* of 99.1, 130.6, 144.6, 144.8, 155.3, 156.1 and 156.4"[21].

› Compound 3 (Quercetin):

^{1}H NMR (400 MHz, DMSO-d_6): δ "7.74 (1H, d, *J* = 2.1 Hz, H-2'), 7.62 (1H, dd, *J* = 8.3, 2.1 Hz, H-6'), 6.88 (1H, d, *J* = 8.3 Hz, H-5'), 6.39 (1H, d, *J* = 2.0 Hz, H-8), 6.18 (1H, d, *J* = 2.0 Hz, H-6). ^{13}C NMR (100 MHz, DMSO-d_6): δ d 93.2 (C-8), 98.0 (C-6), 102.9 (C-10), 115.0 (C-2'), 115.4 (C-5'), 119.8 (C-6'), 121.8 (C-1'), 135.5 (C-3), 144.9 (C-3'), 146.7 (C-2), 147.5 (C-4'), 156.0 (C-9), 160.6 (C-5), 163.8 (C-7), 175.7 (C-4)"[22].

› Compound 4 (Quercetin-3-O-β-D-glucopyranoside):

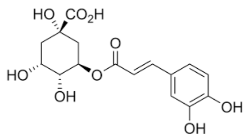
^{1}H NMR (400 MHz, DMSO-d_6): δ "7.70 (1H, d, *J* = 1.9 Hz, H-2'), 7.68 (1H, dd, *J* = 8.5, 1.9 Hz, H- 6'), 6.86 (1H, d, *J* = 8.5 Hz, H-5'), 6.36 (1H, d, *J* = 2.2 Hz, H-8), 6.17 (1H, d, *J* = 2.2 Hz, H-6), 5.32 (1H, d, J = 7.3 Hz, H-1"), 3.85-3.30 (6H, sugar protons).^{13}C NMR (100 MHz, DMSO-d_6): δ 156.2 (C-2), 133.3 (C-3), 177.5 (C-4), 161.2 (C-5), 98.7 (C-6), 164.1 (C-7), 93.5 (C-8), 156.3 (C-9), 104.0 (C-10), 121.2 (C-1'), 115.5 (C-2'), 144.8 (C-3'), 148.5 (C-4'), 116.2 (C-5'), 121.6 (C-6'), glucose: 100.8 (C-1"), 74.1 (C-2"), 76.5 (C-3"), 70.0 (C-4"), 77.6 (C-5"), 61.0 (C-6")"[23].

› Compound 5 (Kaempferol-3-O-β-D-glucopyranoside):

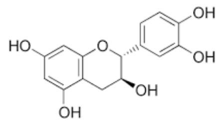
^{1}H NMR (400 MHz, DMSO-d_6): δ "8.03 (2H, d, *J* = 8.6 Hz, H-2', 6'), 6.87 (2H, d, *J* = 8.6 Hz, H-3', 5'), 6.36 (1H, d, *J* = 2.3 Hz, H-8), 6.17 (1H, d, *J* = 2.3 Hz, H-6), 5.33 (1H, d, *J* = 7.6 Hz, H-1"). ^{13}C NMR (100 MHz, DMSO-d_6): δ 178.1 (C-4), 164.1 (C-7), 161.2 (C-5),160.2 (C-4'), 157.6 (C-2), 157.2 (C-9), 134.0 (C-3), 130.9 (C-2'), 130.9 (C-6'), 120.9 (C-1'), 114.7 (C-3'), 114.7 (C-5'), 104.2 (C-10), 98.7 (C-6), 93.5 (C-8), 102.7 (C-1"), 74.4 (C-2"), 77.0 (C-3"), 70.0 (C-4"), 76.7 (C-5"), 61.2 (C-6")"[24].

› Compound 6 (Luteolin-8-C-β-D-glucopyranoside):

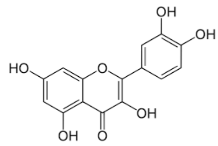
^{1}H NMR (400 MHz, DMSO-d_6) δ: "13.15 (1H, s, 5-OH), 10.82-9.15 (3H, s, 3′, 4′, 7′-OH), 7.52 (1H, dd, J = 2.2, 8.3 Hz, H-6′), 7.45 (1H, d, J = 2.2 Hz, H-2′), 6.90 (1H, d, J = 8.3 Hz, H-5′), 6.65 (1H, s, H-3), 6.56 (1H, s, H-6), 5.05 (1H, d, J = 7.0 Hz), 3.30-3.90 (sugar protons, m). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 164.16 (C-2), 102.41 (C-3), 182.03 (C-4), 160.47 (C-5), 98.33 (C-6), 162.80 (C-7), 104.65 (C-8), 156.00 (C-9), 104.00 (C-10), 121.97 (C-1′), 114.07 (C-2′), 145.95 (C-3′), 149.90 (C-4′), 115.78 (C-5′), 119.45 (C-6′), 73.50 (C-1″), 70.90 (C-2″), 78.88 (C-3″), 70.83 (C-4″), 82.04 (C-5″), 61.76 (C-6″)"[25]



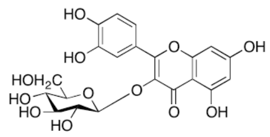
Chlorogenic acid



Catechin



Quercetin



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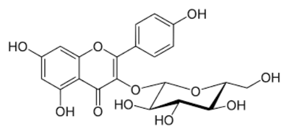
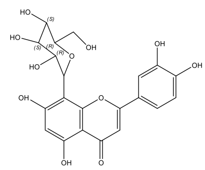


Kaempferol-3-O-β-D-glucopyranoside



Luteolin-8-C-β-D-glucopyranoside

Second part: Biological studies

Cytogenetic studies

Micronuclei in bone marrow

The results (Table 2) indicated a significant increase of MPEs after cisplatin treatment where the percentage reached 8.63 ± 0.21 after treatment with the dose 15 mg/ kg in comparing with 3.27 ± 0.40 for the control. It also induced severe toxicity to bone marrow cells evidenced by an increase in the percentage of PEs/total counted cells. On the other hand *Ficus carica* alleviated such percentages in a dose-dependent manner. Significant protection was recorded with the highest tested dose.

Chromosomal abnormalities

Table 3 showed that cisplatin at the tested doses (10 and 15 mg/kg) induced highly significant percentage of chromosomal abnormalities in bone marrow cells reached 28.40 ±1.2, 57.40±0.68 respectively compared with 4.4 ±0.51 for control. The results also demonstrated that the combined treatment with *Ficus carica* extract at different doses ameliorated such effect in a dose-dependent manner. Moreover, *Ficus carica* alone (600 mg/kg) had a normal effect as compared with that of the control negative. The majority of aberrations are fragments and/or breaks and metaphases contained more than five aberrations in the same metaphase (MA). Fragmentation may be included in the whole set of chromosomes. Metaphases with multiple aberrations represent a pronounced percentage (approximately 32% of the total aberrant metaphases).

In mouse spermatocytes, Table 4 showed that both doses of cisplatin and also *Ficus carica* at the dose 600 mg/kg had no significant effect on chromosomal abnormalities and their effect is nearly the same as control negative.

Quantitative gene expression

Table 5 & Figure 1 demonstrated that cisplatin induced a significant increase in the expression of iNOS gene compared to negative control (P<0.001). *Ficus carica* had normal effect on iNOS gene the same as control negative (P= 0.189). The results also demonstrated that *Ficus carica* at different doses (in combined treatment fig + cisplatin) alleviated the over expression of iNOS gene compared to cisplatin group especially with the doses 400 and 600 mg/kg (P<0.001). No

Table 2: Frequency of polychromatic erythrocytes (PEs) and PEs with micronuclei induced in mouse bone-marrow cells after treatment with cisplatin and *Ficus carica* extract.

Treatment and doses (mg/ kg)	PEs		MPEs	
	NO.	Mean ± S.E	NO.	Mean ± S.E
I. Control (non-treated)	550	5.50 ± 0.19 [a]	18	3.27 ± 0.40 [a]
II. Control (plant, 600mg/kg)	512	5.12 ± 0.14 [a]	17	3.32 ± 0.41[a]
III, IV. Cisplatin				
10 mg/kg	936	9.36 ± 0.40 [b]	64	6.84 ± 0.47 [b]
15 mg/kg	1622	16.22 ±0.29 [c]	140	8.63 ± 0.21[c]
V-VII. cisplatin + *Ficuscarica*				
Cisplatin + 200 mg/kg	1436	14.36 ± 1.37 [c]	94	6.54 ± 0.50 [b]
Cisplatin + 400 mg/kg	1004	10.04 ± 0.65[b]	68	6.77 ± 0.74 [b]
Cisplatin + 600 mg/kg	866	8.66 ± 0.68 [b]	45	5.19 ± 0.96 [b]

Number of examined nucleated cells = 2000/mouse (5 mice / group). The values having different superscript letters in each column are significantly different from one another as calculated by ANOVA. The data were presented as mean ± S.E. (n=5).

Table 3: Frequency of chromosomal aberrations induced in bone marrow cells after treatment with cisplatin and *Ficus carica* extract.

Treatment and doses	Abnormal metaphases		No. of different types of chromosomal aberrations				
	NO.	Mean ± S.E	Chromat.and/or chromos. Gap	Frag. and / or break	Frag. and or break +gap	MA	RT/or Ring chromosome
I. Control(non-treated)	**22**	**4.4±0.51[a]**		**7**	**-**	**-**	**-**
II. Control plant (600 mg/kg)	**23**	**4.6± 0.68[a]**	**11**	**12**	**-**	**-**	
III-IV. Cisplatin							
10 mg/kgb.wt	**142**	**28.40±1.2[d]**	**16**	**44**	**8**	**74**	**-**
15 mg/kgb.wt	**287**	**57.40±0.6[ef]**	**12**	**113**	**63**	**91**	**8**
V-VII. Cisplatin+ *Ficus carica*							
+ 200 mg/kg	**216**	**43.2±1.32[de]**	**33**	**105**	**18**	**60**	**-**
+400 mg/kg	**191**	**38.2±2.15[de]**	**24**	**108**	**9**	**50**	**-**
+600 mg/kg	**134**	**26.8±1.91[c]**	**16**	**85**	**9**	**24**	**-**

Number of examined metaphases = 500 (100 metaphases/mouse, 5 mice/group). Chromat. = Chromatid; Frag. = fragment, MA=Multiple aberrations. RT= Robertsonian translocation.The values having different superscript letters in each column are significantly different from one another as calculated by ANOVA. The data were presented as mean ± SE (n=5).

Table 4: Frequency of chromosomal abnormalities in primary spermatocytes of male mice induced after treatment with different doses of cisplatin and Ficus carica extract.

Treatment and doses	Abnormal metaphases		No. of different types of chromosomal abnormalities	
	NO.	Mean ± S.E	XY- U	A- U
I-Control (non-treated)	19	3.8 ± 0.58[a]	12	7
II- Control plant (600 mg/kg)	16	3.2 ± 0.37[a]	10	6
III, IV. Cisplatin				
10 mg/kg b.wt	20	4.0 ± 0.45[a]	15	5
15 mg/kg b.wt	22	4.4 ± 0.51[a]	13	9

Number of examined metaphases=500/mouse (5 mice/group); XY-U= XY-univalent; A-U=. Autosomal univalent.

Table 5: Quantitative iNOS and NF-kβ gene expressions after treatment with cisplatin and *Ficus carica* extract.

Treatment and doses	Mean	Std. Deviation	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
INOS gene						
I. Negative Control	0.76	0.05	0.637	0.89	0.72	0.82
II. Cisplatin	2.98	0.13	2.65	3.32	2.87	3.13
III. *Ficus carica*	0.94	0.028	0.87	1.015	.91	0.96
IV. Cisplatin +fig (200 mg)	2.20	0.053	2.07	2.339	2.16	2.26
V. Cisplatin + fig(400 mg)	1.75	0.139	1.406	2.097	1.60	1.87
VI. Cisplatin + fig (600mg)	1.69	0.034	1.61	1.78	1.66	1.73
NF-kβ gene						
I. Negative Control	1.20	0.056	1.065	1.34	1.14	1.25
II.Cisplatin	4.32	0.167	3.9	4.74	4.17	4.50
III. *Ficus carica*	1.34	0.25	.72	1.97	1.12	1.62
IV. Cisplatin + fig (200mg)	3.15	0.357	2.27	4.047	2.82	3.53
V. Cisplatin + fig (400mg)	2.35	0.33	1.526	3.18	1.99	2.63
VI. Cisplatin + fig (600mg)	1.96	0.268	1.298	2.63	1.80	2.28

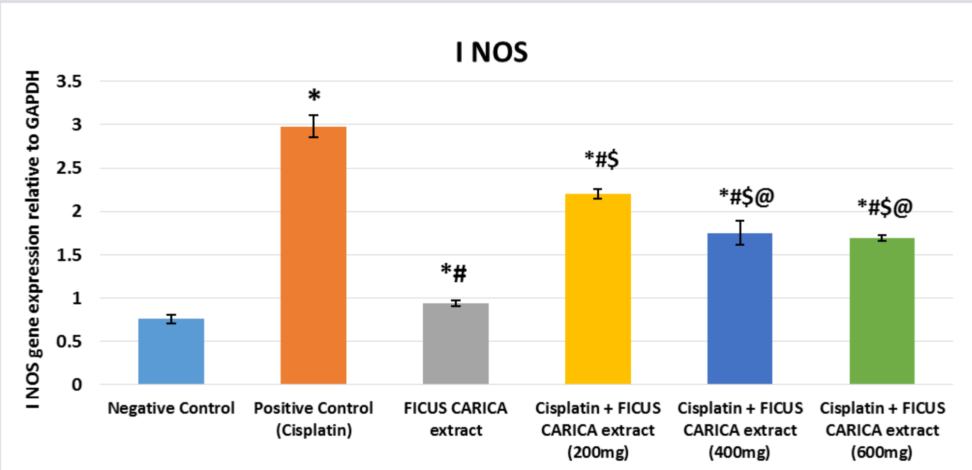


Figure 1: Quantitative iNOS gene expression induced after treatment with cisplatin and *Ficus carica* extract.
Data were expressed as Mean ± SD, p value <0.05 was significant
(*) Denotes significant difference *versus* negative control group
(#) Denotes significant difference *versus* positive group.
($) Denotes significant difference *versus Ficus carica* extract groups.
(@) Denotes significant difference *versus* Cisplatin + *Ficus carica* extract groups.

significant difference between the effects of the two doses 400 and 600 mg/kg fig (P=0.963).

Concerning the gene NF-kB the results (Table 5, Figure 2) demonstrated an over expression of the gene after cisplatin treatment (15 mg/kg) compared to the negative control (P<0.001) and that there was no significant difference between *Ficus carica* (plant control) and the negative control (P=0.963). The results also demonstrated the protective role of fig. When fig was given with cisplatin the expression of NF-kB gene decreased to a good extent especially with the doses 400 and 600 mg/kg in comparison with cisplatin alone (P<0.001) but the expression was still elevated in comparison with the control negative (P< 0.001, 0.002, 0.034) after the three tested doses of fig respectively.

Histopathological studies

The histological observations of the testis from control and *Ficus carica* groups showed normal appearance of spermatogenic and interstitial cells and seminiferous tubules (Figure 3A). Mice treated with CP showed many histopathological alterations in spermatogenic cells, pyknotic nuclei appearance, dilatation and congestion of blood vessels with marked reduction of spermatozoa in the lumen of the seminiferous tubules (Figure 3B). In combination with cisplatin and fig, it can be detected that the lower and the medium dose of the plant gave a partial improvement of spermatogenic cells. Pyknotic nuclei and many alterations of the spermatogenic process were still observed (Figure 3C). With the highest tested dose of fig, all histopathological changes were reduced and spermatogenic cells were improved to a good extent while the interstitial congestion was still found (Figure 3D). Immunohistochemistry of TNF-α:

Undetectable immunoreactivity of TNF-α in control or plant treated mice was observed (Figure 4A). In the cisplatin group, intense of TNF-α expression was detected mainly in seminiferous tubules of the testis (Figure 4 B). Mice treated with cisplatin and plant at the three tested doses showed moderate to weak TNF-α immunoreactivity (Figure 4 C & D). Dose -dependent protection was recorded.

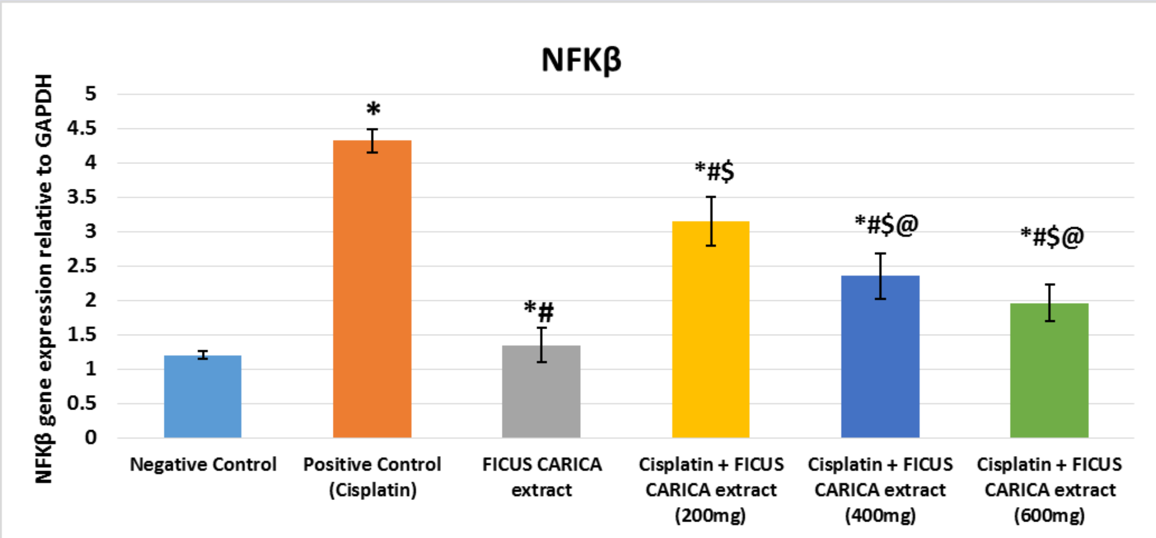


Figure 2: Quantitative NF-kβ gene expression induced after treatment with cisplatin and *Ficus carica* extract.
Data were expressed as Mean ± SD, p value <0.05 was significant
(*) Denotes significant difference *versus* negative control group
(#) Denotes significant difference *versus* positive group.
($) Denotes significant difference *versus Ficus carica* extract groups.
(@) Denotes significant difference *versus* Cisplatin + *Ficus carica* extract groups

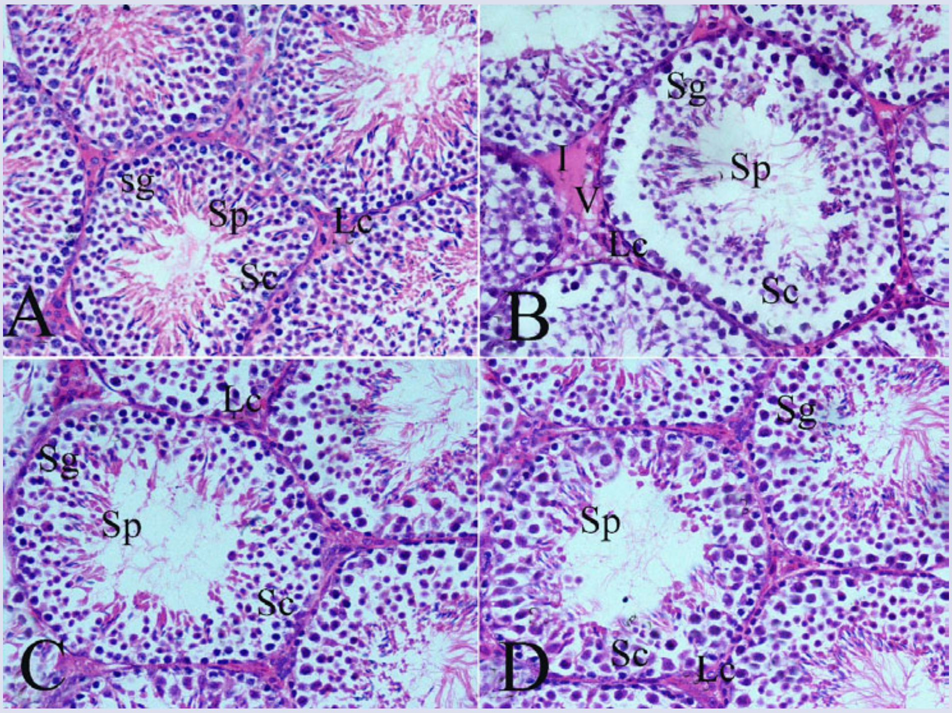


Figure 3: Light micrographs of sections of mice testis induced after treatment with cisplatin and *Ficus carica*.

A. Control group showing normal seminiferous tubules, including spermatogonia (Sg), spermatocytes (Sc) and spermatids (Sp) Leydig cells (Lc).

B. Cisplatin group showing marked reductions of (Sg), (Sc), spermatozoa produced in the lumen of the seminiferous (Sp), vacuolated (V) pyknotic (Lc), interstitial congestion (I).

C. Cisplatin and the medium dose of plant showing mild reductions of (Sg), (Sc), and spermatozoa produced in the lumen of the seminiferous (Sp), with pyknotic (Lc).

D. Cisplatin and the high dose of plant showing restored alteration of (Sg), (Sc) and spermatozoa produced in the lumen of the seminiferous (Sp), with pyknotic (Lc) (H & E staining- ×400 magnification).

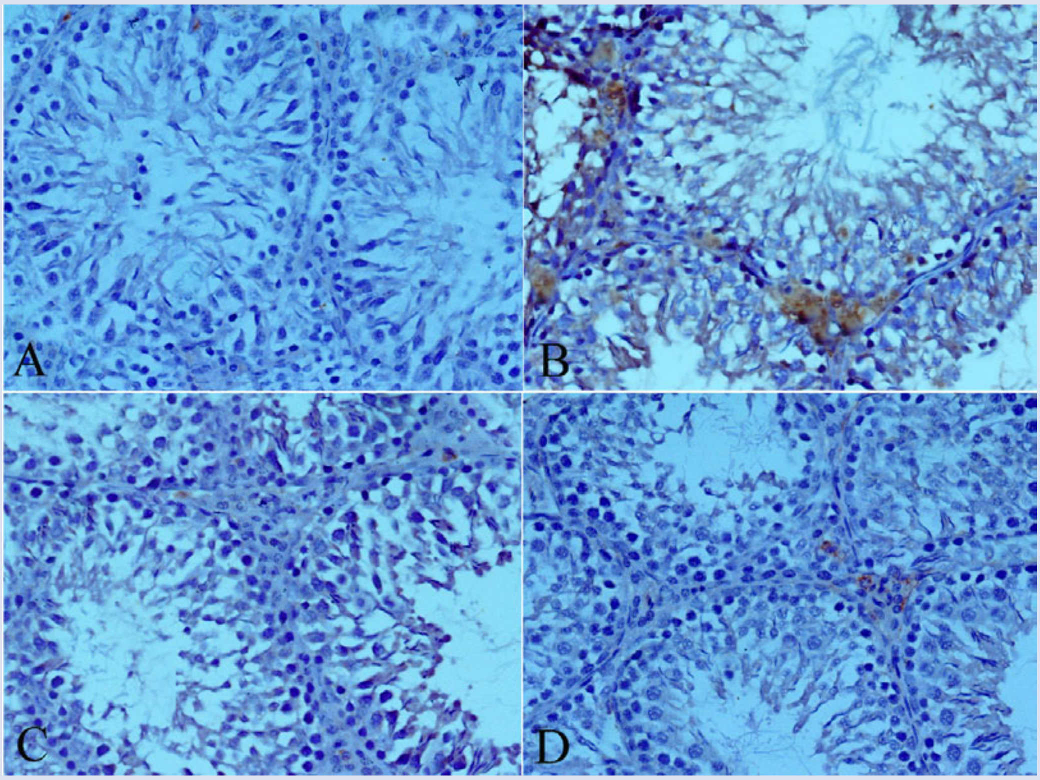


Figure 4: Immunohistochemical expression of TNF-α induced after treatment with cisplatin and *Ficus carica*

A Negative control group showing undetectable immunoreactivity

B. Cisplatin group showing intense immunoreactivity

C. Cisplatin + the medium dose of plant showing mild immunoreactivity

D. Cisplatin + the high dose of plant showing weak immunoreactivity (TNF-α immune staining- × 400).

DISCUSSION

Cisplatin (CP) is a platinum-based standard antineoplastic drug that is used against different types of solid tumors and neoplasms. Like other chemotherapeutic drugs CP possesses many side effects in clinical uses and experimental animal studies. The use of CP for clinical purposes is limited by its side effects[12].

Plants have been an important part of sophisticated traditional medicine for thousands of years. Nowadays, scientific researches have shown that many plants can induce anti-mutagenic, chemo-protective, anti-inflammatory and antioxidant activities[1]. In the present study, the protective efficacy of *Ficus carica* leaves methanol extract was investigated against genotoxicity, liver gene expression and reproductive toxicity of cisplatin. Separation and identification of some active constituents of fig and their biological bioactivity were discussed.

The genetic endpoint was discussed in the present work by detecting micronuclei in polychromatic erythrocytes (MPEs) of bone marrow, chromosomal aberrations (CAs) in bone marrow and primary spermatocytes and also its effect on the expression of the liver genes TNF-α, iNOS and NF-kB was demonstrated. The detection of MPEs is one of the most commonly used endpoints in the field of genetic toxicology[14]. The results demonstrated that cisplatin induced significant percentage of MPEs which increased by dose. Also it induced highly significant incidence of chromosomal aberrations in bone marrow cells especially with the highest tested dose. Generally, it was accepted that MPEs are formed as a consequence of chromosome breakage/fragment or aneuploidy. Moreover, micronucleus assay is considered as a sensitive method for determining DNA damage induced after chemotherapy or radiotherapy[26]. The present results also showed that cisplatin induced cytotoxicity to bone marrow cells by increasing the percentage of PEs in relation to normochromatic erythrocytes. The present results coincide with the findings of other authors who demonstrated that cisplatin induced myelosuppression in bone marrow cells and induced different forms of genotoxicity *e.g* micronuclei, chromosomal aberrations in mouse bone marrow and DNA damage[10, 27]. In the present work *Ficus carica* extract administrated to cisplatin–treated mice reversed part of these alterations in a dose–dependent manner. Anti-mutagenic effect of *Ficus carica* extract was previously demonstrated against mutations induced by N-metil-N'-nitro-N-nitrozoguanidin (MNNG) in *Vicia faba* cells, chlorophyll mutations in *Arabidopsis thaliana* and NaF induced mutability in rat marrow cells[28]. It was also reported to have the ability to decrease the frequency of spontaneous and induced- $_{\gamma}$ ray chromosome abnormalities in meristematic cells of *Vicia faba* and bone marrow cells of mice[29].

On the contrary, cisplatin did not induce any significant effect on chromosomal aberrations in mouse primary spermatocytes (germ cells) after treatment at the two dose levels examined in comparing to the control. It was previously reported that chromosome damage in germ cells is an indirect method to assess transmissible genetic damage[15]. The results obtained gave cisplatin an advantage over some other chemotherapeutic drugs which induce high percentage of chromosome defects in germ cells eg: 5-florouracil[30]; Etoposide[31]; methotrexate and tamoxfine[32]; vinblastine[33] and cyclophosphamide[34].

Histopathological investigation of the testes revealed many deleterious effects after cisplatin treatment that produced a pronounced alteration of spermatogenesis with marked reduction of spermatozoa in the lumen of seminiferous tubules. These histopathological changes were reversed with dose-dependent manner in mice treated with *Ficus carica* extract. The mentioned results coincide well with the findings of other authors who demonstrated that cisplatin had impaired spermatogenesis and resulted in gonadal dysfunction, altered Leydig cells and antioxidant enzyme status[12]. Also temporary or permanent azoospermia are other forms of side effects associated with cisplatin treatment. Such side effects are attributed to oxidative and nitrosative damage generated by CP[35]. All of these defects are associated with impairment in reproductive function after CP treatment.

Concerning to the effect of cisplatin on the gene level. The results obtained from using QRT-PCR indicated an overexpression of the two genes NF-kB and iNOS after treatment with cisplatin (15 mg/kg). It is well documented that the nuclear factor-κB (NF-κB) family of transcription factors controls the expression of genes involved in many critical physiological responses such as inflammatory responses, differentiation, proliferation, cell adhesion and apoptosis[36]. Constitutively activated NF-κB transcription factors have been associated with several aspects of tumorigenesis, including promoting cancer-cell proliferation, preventing apoptosis, and increasing tumor's angiogenic and metastatic potential. Activation of the NF-κB plays a role in inflammation through the induction of transcription of several pro-inflammatory genes[37]. Oxidative stress plays a critical role in NF-kB activation by diverse agents. This hypothesis is based on that oxidant such as H_2O_2 can activate NF-kB[38] and that drugs with antioxidant properties have an inhibitory effect on the gene[39]. The results of the present work demonstrated that *Ficus carica* in the combined–treated groups alleviated the overexpression of the NF-kB gene.

Inducible nitric oxide synthase (iNOS) gene is located on chromosome 17 and has been implicated in a wide variety of diseases. This gene encodes a nitric oxide synthase (NOS) which is expressed in liver and induced by a combination of lipopolysaccharide and certain cytokines. The nitric oxide synthase enzyme forms nitric oxide that besides being a signaling molecule plays an important role in host immune response. Nitric oxide is a reactive free radical which acts as a mediator in many biological reactions including neuro-transmission, antimicrobial and antitumor activities[40]. Increased levels of NO have cytotoxic and DNA-damaging effects. In inflammatory states, nitric oxide may be synthesized from precursor L-arginine *via* inducible NO synthase (iNOS) in large amounts for prolonged periods of time. When NO acts as an effector molecule under these conditions, it may be toxic to cells by inhibition of iron-containing enzymes or initiation of DNA single-strand breaks[41] .The results showed that *Ficus carica* at the highest tested dose reversed the over expression of iNOS gene to near the normal value. TNF-α gene is critical in the regulation of invasion, angiogenesis, and tumor metastasis. Immunohistochemically investigation in the tissues is a surrogate of mutational analysis. In the present study, intense of TNF-α expression was detected mainly in seminiferous tubules of the testis after CP treatment. *Ficus carica* at different doses relieve this effect.

On referring to the mechanism of action of cisplatin and *Ficus carica*, it is generally known that CP is an alkylating agent that kills cells by several mechanisms including DNA damage in the form of single and double-strand breaks that are poorly repaired by nuclear repair enzymes[9], production of reactive oxygen species (ROS) which inhibit the antioxidant defense systems and the induction of apoptosis[12]. As with all chemotherapeutic drugs, CP kills both cancerous and normal cells. Therefore, it comes with severe side effects such as nephrotoxicity, infertility, neurotoxicity, and ototoxicity[9]. Genotoxicity induced by some anticancer drugs in normal tissues might also result in secondary malignancies in patients especially after extended remission[1].

Ficus carica (Moraceae genus) is an important genetic resource due to its high nutritional and economic values. Fig leaves are known to be rich in flavonoids[42]. This opinion is supported by the results obtained in the present work where the total flavonoid content reached 59.67 mg/g extract. Flavonoids are potent antioxidants that inhibit lipid peroxidation[43], protect the tissues from free radicals by direct scavenging ROS, reactive nitrogen species (RNS), and activating antioxidant enzymes[1]. Fig is also an excellent source of phenolic compounds (represents 63.37 mg/kg extract in the present results), whereas red wine and tea, which are two good sources of phenolic compounds, contain phenols lower than those in fig[44]. Arvaniti *et al.*[45] reported that antioxidant capacity of figs is highly correlated with their amount of phenolic compounds. Phenolic compounds can act as antioxidants by different ways: reducing agents, hydrogen donators, free radical scavengers, singlet oxygen quenchers, and so forth[2]. In-depth chromatographic investigation of the active constituents of *Ficus carica* extract resulted in the identification of Catechin, Luteolin-8-C-β-D-glucopyranoside, Quercetin, quercetin-3-O-β-d-glucopyranoside, Chlorogenic acid and Kaempferol-3-O-β-D-glucopyranoside. Catechins were previously demonstrated to have protection against oxidative DNA damage[46]; chlorogenic acid and quercetin have antioxidant and anti-inflammatory properties[47, 48]. Quercetin-3-O-β-d-glucopyranoside was reported as a cytoprotective agent by decreasing in the generation of reactive oxygen species[49]; Kaempferol-3-O-β-D-glucopyranoside recorded scavenging activities in the ONOO and DPPH tests[50] and Luteolin has antitumor therapeutic effects[51]. Previously some phenolic compounds with reported pharmacological properties have been isolated from fig leaves, namely furanocoumarins like psoralen and bergapten[52]; flavonoids like rutin[53] ; phenolic acids like ferrulic acid and also phytosterols like taraxasterol[54].

In conclusion, this study shows that Ficus carica has important role in mitigating Cisplatin-mediated genotoxicity and reproductive impairments. Ficus carica is rich with many bioactive constituents and may serve as a promising candidate for future development.

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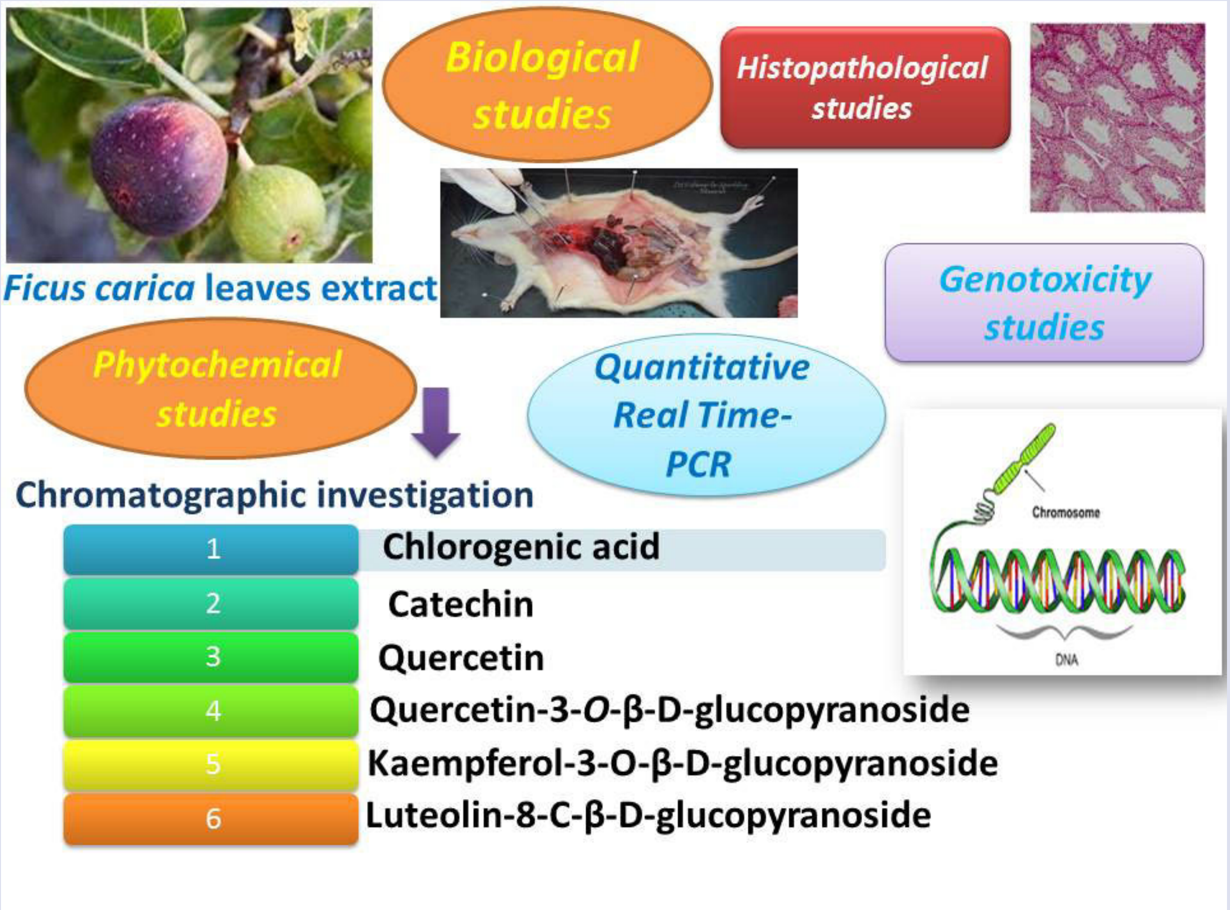
GRAPHICAL ABSTRACT



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