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Evaluation of ethanol extract of *Morus nigra* L. as an inhibitory agent for DNA-Advanced glycation end product (DNA-AGEs)

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In the developing countries there is a direct correlation between the amount of consumed foods containing starch and the increase in metabolic diseases. In contrast to glucose, fructose cannot be detected in the blood by insulin. Thus, it participates in lipogenesis and increases intracellular lipid accumulation.Reducing sugars results in the development of AGEs (Advanced Glycation End-Compounds) in biological macromolecules as well as some reactive products. These products can cause tissue damage by accumulating in the pathogenesis of a number of diseases by various mechanisms.AGEs can damage antioxidant systems by increasing ROS (reactive oxygen species). In this study, pBR322 DNA was incubated with different concentrations of fructose for 5 days. Damage to the structural system of fructosylated DNA was detected by an increase in fluorescence intensity and hyperchromicity. For five days, fructosylated DNA was treated with varying quantities of *Morus nigra* L. soxhlet extract and quercetin, and its impact on DNA structural damage was noted. The reduction in hyperchromicity and fluorescence intensity revealed the protective impact of quercetin and *M. nigra* L. extract on DNA. It has been found that the extract of *M. nigra* L. and quercetin both scavenge free radicals and reduce fructose-induced DNA damage.

Keywords: DNA fructosylation, DNA oxidation, DNA-AGEs, *Morus nigra* L., Reactive oxygen species

Glycation is a non-enzymatic reaction that occurs under conditions of hyperglycemia and aging. The reducing ends of reactive free sugars (*e.g.*, glucose, fructose and galactose) are covalently attached to the amino acid residue of the protein, thereby forming glycated products[1]. The non-enzymatic and h-long reactions of the keto-aldehyde group of carbohydrates and the amine groups of biological macromolecules (DNA, lipids and proteins) result in the formation of highly unstable Schiff bases. Composed shiff bases forms more stable amadori products and dicarbonyl compounds within days with rearrangement, dehydration and cyclization reactions (all reactions up to this step are reversible reactions)[2]. Amadori products are aldehyde derivatives through various sequential reactions (enolization and strecker degradation) can form melonoidins. Both schiff bases and Amodori products and dicarbonyl compounds form Reactive Oxygen Species (ROS) and Advanced glycation end products (AGEs) irreversibly at the end of weeks/months[3]. The reactions leading to the formation of AGEs have been known since 1912 with the work of Maillard[4].

AGEs were first studied when HbA1c was identified in diabetic individuals in 1968. It can originate from the non-enzymatic biological alteration of substances with amine groups, the production of carbohydrates with aldehyde or keto groups, the formation of Amadori products during glycation, and other processes. It is important to monitor HbA1c levels in all diabetic patients and especially in young people. Hyperglycemia causes general protein glycation and leads to protein cross-links. It causes physiological activities that will result in disaster over time[5].

HbA1c monitoring is an indicator of insulin adherence. HbA1c is an advanced glycation end product. In a study for cooking method and AGE level linkage, various cooking techniques were used in 250 types of food. The method with the highest AGE level was determined as frying and barbecue. The lowest AGE level was detected in boiled food[6]. The

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Abbreviations: 3-DG, 3-deoxyglucazone; AG, Aminoguanidine; AGEs, Advanced Glycation end products; BHA, Butylatedhydroxyanisole; BHT, Butylatedhydroxytoluene; CEL, Carboxyethyl lysine; CML, Carboxymethyl lysine; DM, Diabetes Mellitus; DNA, Deoxyribonucleic acid; EDTA, Ethylenediamine tetra acetic acid; F, Fructose; G, Glucose; GAE, Gallic acid; GO, Glyoxal; GSH, Glutathione; *M. n.* L., *M. nigra* Leaves; MDA, Malondialdehyde; ME, Maceration; MG, Methylglyoxal; Que, Quercetine; RAGE, Receptor for AGEs; RCS, Reactive carbonyl species; ROS, Reaktive oxygen species; SE, Soxhlet; SOD, Superoxide dismutase; TCA, Trichloroacetic acid; US, Ultrasound

process of cooking foods at dry heat and fast (grilling, barbecue, frying…) is called glycation/browning or caramelization. This process improves the flavor, smell and color of food, but accelerates the formation of AGEs species. While the heating process increases the safety of the food, the addition processes and the main proteins in the foods can cause the structure to deteriorate. The first step in the formation of aroma in foods is thanks to aldehyde derivatives. This step is also the third step of the Maillard reaction. In order to significantly reduce the level of AGEs it is necessary to cook foods at low temperature in a short time with plenty of water or steam.

AGEs can form a variety of complex labile structures in diabetes classes. AGE formation can accelerate hyperglycemia and together with it prepares the ground for the formation of various diseases. AGEs can modifies protein and lipid groups. Reactive oxygen species formed during glycation disrupt the antioxidant system through advanced glycation end products. Thus oxidative-chronic stres it causes many diseases, especially diabetes and cancer. Glycation of DNA results in the formation of DNA-AGEs. This causes mutations such as DNA depurination, strand breakage, insertion and deletion. Therefore DNA-AGEs the loss of genomic integrity that occurs during aging can lead to cancer and other diseases[7].

AGE weakens the elasticity of the skin and activates aging. If it is close to the skin, it causes skin cancer. It is activated by UV modifying proteins and causing DNA damage again. Glycation proteins in the skin function equally as photoactive in photoaging and photo cancer. If these UV active products are in the eye they can trigger or create eye disorders and skin disorders in the skin[8].

However recent studies have confirmed that not only glucose, but also ribose and fructose, are associated with diabetes as they cause high concentrations of glucose in metabolism. Diabetes mellitus can develop into a range of complications such as retinopathy, nephropathy, poor bone strength, neuropathy, atherosclerosis and CVD[9-13].

One of the main causes of obesity in the world especially in the USA is fructose/glucose syrup. Excessive-uncontrolled intake of carbohydrates causes the Maillard Reaction[14-16]. The advanced glycation end products receptor (RAGE) is a new protein that is increasingly being investigated in the pathogenesis of type 1 diabetes (T1D). AGEs on the cell surface can bind to RAGE (AGE-binding receptor) and cause metabolic changes by causing activation in various signaling pathways. RAGEs can be found in smooth muscle cells and endothelial cells[17].

As the complexity of their involvement is far beyond simple studies of the isolated signaling pathways, further research should aim to expand the knowledge on AGE formation, receptors and competition with other ligands (*e.g*., in the case of multi ligand receptors such as RAGE or Stab2). The structural basis of AGE interaction with receptors is limited, and providing the experimental structures of these complexes would undoubtedly facilitate the understanding of their still enigmatic roles[18]. Natural products of plant origin are the main quarry for promising discoveries. Easy availability, low cost and minimal side effects make plant-based preparations the main key players. In addition, many plants provide a rich source of nutrients[19-20]. Antiglycation activities of phenolic compounds in the structure of plants were investigated. In the process of antiglycation, the most common mechanism of action of antioxidants is to scaveng free radicals[21].

Mulberry leaves effective against high blood pressure, throat infections, irritations and inflammations, and residues from alcohol. In studies conducted in the last decade, mulberry tea consumption has increased due to the hypoglycemic, antidepressant, antioxidant and hepatoprotective effects of black mulberry leaf[22-23].

Black Mulberry *M. nigra* L. (Moraceae) has various uses in traditional medicine. The major organic acid in the black mulberry leaf content is citric acid, as well as malic acid and ascorbic acid, gallic acid and naringenin. The antioxidant capacity (oxygen radical absorbance capacity) (ORAC) of black mulberry varies between 13.95-16.01 µmol/g. Compared to other fruits, it has the highest inhibition effect against O_2, H_2O_2 and OH radicals[24].

It is known that the AGEs process plays an major role in the development of complications in diabetes[25]. In studies on this subject protein glycation has been studied more. There are limited studies on DNA glycation. For this reason in this study a plant known to have antidiabetic, anticancer and antioxidant properties in the DNA-fructose system was desired to be studied.

Pharmaceutical precursors in herbal sources are cheaper and have less side effects compared to synthetic sources. This is the first study of the inhibition of *Morus nigra* L. ethanol extract and quercetin on fructosylation DNA. The goal of the study was to look into the anti-

glycation and antioxidant properties of quercetin and *M. nigra* L. against free radical- and fructose-induced DNA damage. By assessing the inhibitory activity in the development of AGEs, new AGE inhibitors from natural sources are to be found.

Materials and Methods

Materials

Plant type used

The *M. nigra* L. used in the study were collected from Diyarbakır in May 2017 and it was obtained from Dicle University, Field Crops Department, Agriculture Faculty Diyarbakır, Turkey. The plant was identified as *M. nigra* L. by Dr. Selçuk Ertekin, Dicle University, Department of Biology, Faculty of Science, Diyarbakır, Turkey.

Chemicals used

Gallic acid, ethanol, 1,1-diphenyl-2-picryl-hydrazil (DPPH), butylatedhydroxytolue (BHT), butylatedhydroxyanisole (BHA), α-tocopherol, Quercetin, foline & ciocalteu's phenol reagent, sodium carbonate, iron-2-chloride, iron-3-chloride, ethylenediaminet-etraacetic acid (EDTA), hydrogen peroxide, ferrosine, ferricyanide, trichloroacetic acid (TCA), KH_2PO_4, K_2HPO_4 deoxyribose, 2-thiobarbituric acid (TBA), sodium hydroxide, pBR 322 plasmid DNA, ascorbic acid, agarose, tris base, comassie brillant blue R, aminoguanidine, D-fructose, metformin were commercially available from Aldrich and Merck.

Methods

Extraction procedures

The extraction of volatile fraction from *M. nigra* L. was performed using three different methods, and each test was carried out in triplicate. Ethanol (96%) was used as solvent.

Maceration method

Maceration (ME) is one of the conventional methods of extraction that is very simple and the cheapest because it only requires a simple container as the place for extraction, but this method requires a long time for the extraction process[26]. 20 g of dried *M. nigra* L. powder were extracted three times with 96% aqueous ethanol (500 mL) for a total extraction time of 24 h. The obtained liquid was filtered and concentrated by rotary and kept at 4°C for further analyses[27].

Soxhlet method

Soxhlet extraction (SE) was used as a conventional extraction technique. A measured amount of the defatted dried ground material (20 g) dried *M. nigra* L. was placed in a cellulose thimble and 500 mL ethanol was for extraction. The total extraction time was 6 h and temperature was set according to boiling point of the solvent[28]. The crude extract obtained was dried using rotary evaporator and yield was calculated.

Ultrasound method

20 g of dried *M. nigra* L. material with 200 mL 96% ethanol was allowed to stand 8 h and then placed into the reactor. Then the applied for 30 min operating at a frequency of 25 kHz and 45°C[29]. The crude extract obtained was dried using rotary evaporator and yield was calculated.

The remaining water extract was frozen in liquid nitrogen and freeze-dried in a Christ Alpha 1-2 LD plus lyophilizer under −86°C at 0.0021 mbar pressure for 24 h[30]. *M. nigra* L. maceration extract (8.52 g) was obtained with a yield of 42.6%, soxhlet extract (8.58 g) was obtained with a yield of 42.9%, ultrasound extract (9.39 g) was obtained with a yield of 46.95%. The lyophilized solids were stored at −20°C until use.

Total phenolic, flavonoid and DPPH radical scavenging activities of the extracts obtained as a result of three extraction methods were investigated. It was discovered that the extract prepared using the soxlet method shown superior performance in the DPPH radical scavenging, total phenolic, and total flavonide activities. Deoxyribose assay, DNA fructosylation, DNA oxidation, fructosamine test, UV and fluorescence studies were carried out with the extract obtained from extraction by SE method.

Total phenolic component

Total phenolic content was measured according to the method of with gallic acid as the standard[31]. The absorbance was read at 765 nm using the UV/Visible spectrophotometer. Absorbance values against increasing concentrations of gallic acid were plotted and the following equation was obtained.

Absorbance (A) = 0.00095 x Gallic acid (μg) (R^2: 0.99)

The total phenolic content was calculated from the gallic acid standard curve and expressed as microgram of gallic acid equivalent per 1 mg sample (μg GAE/ 1 mg).

Total flavonoid component

Total flavonoid content of ethanol-water (1:1) was extract determined by the $Al(NO_3)_3$ colorimetric method described with some modifications[32]. The absorbance at 415 nm was read after shaken for 30 s in

spectrophotometer. (Varian, 100 Bio UV/Visible Spectrophotometer). Absorbance values against increasing concentrations of quercetin were plotted and the following equation was procured.

Absorbance (A) = 0.00527 x QUE (µg) (R^2: 0.99)

Quercetin was used as a standard and results were expressed as microgram catechin equivalents per 1 mg of sample (µg Que/mg).

DPPH radical scavenging activity

The free radical scavenging activity of *M. nigra* L. was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH•)[33]. Antioxidants donate their hydrogen to the radical in order to scaveng the DPPH radical. The DPPH radical is free and stable. They become stable by gaining electrons or hydrogen. DPPH radical scavenging activity is more widely used as it provides the opportunity to compare the antioxidant activity in a short time compared to other methods[34]. The basic principle of this method is based on the reduction of absorbance. During the reaction, the color of the mixture changes from purple to yellow. Unpaired electrons on DPPH give maximum absorbance at 517 nm in the visible region. The reaction between the antioxidant molecule and DPPH causes a decrease in the concentration of DPPH in the environment, thus reducing the absorbance. The resulting structure is non-radical DPPH-H.The DPPH• concentration (mM) in the reaction medium was calculated from the following calibration curve determined by linear regression

$$\% I = [(A_{Control} - Sample) / A_{Control}] \times 100$$

OH radical scavenging activity (deoxyribose assay)

The hydroxy radical scavenging activity of the *M. nigra* L. extract used in the study was investigated in the Fe^{2+}/ascorbate/EDTA/H_2O_2 system by the deoxyribose method. Malondialdehyde (MDA) is formed after the hydroxy radical attacks deoxyribose. The resulting MDA reacts with TBA (2-thiobarbituric acid) to form a pink colored MDA-TBA complex[35].

Respectively 100 µL 1 mM EDTA, 10 µL 0.1 mM FeCl3, 100 µL 50 mM H_2O_2, 360 µL 2.8 mM deoxyribose, 1 mL *M. nigra* L. extract (50-500 µg/mL), 330 µL 50 mM pH 7.4 phosphate buffer and 100 µL of 0.3 mM ascorbic acid was used. The mixture was incubated for 1 h in the dark and in a water bath at 37°C. After incubation, 1 mL was taken from the mixtures, respectively; 1 mL of 10% TCA and 1 mL of 0.5% TBA (containing 0.025% BHA in 0.025 M NaOH) were added. It was boiled in a 100°C water bath until the pinkish color turned yellow (approx. 5 min). As soon as color transformation was observed, it was cooled on ice. The absorbance value was measured in UV spectroscopy at 532 nm[36].

There is no plant extract in the negative control and no positive control. The % inhibition values were calculated using the equation below.

$$\% I = [(A_{Control} - Sample)/A_{Control}] \times 100$$

Agarose gel electrophoresis

DNA exists in three forms in agarose gel super coiled form (Form I), single chain broken circular form (Form II), linear form (Form III). These forms act at different speeds in the gel. Form I's charge density is very small so it moves quickly in the gel. Since Form II has less charge density it moves more slowly. Form III has a speed between Form I and Form II[37].

Fructosylation of DNA

Plasmid DNA pBR 322 (0.25 µg/mL) was incubated at five different concentrations of fructose (10, 20, 30, 40, 50 mM) at 37°C for 5 days under steril conditions in 10 mM phosphate buffer saline(pH= 7.4) containing 0.01% sodium azide.

After the specified time of incubation (5 days) unbound fructose was removed by extensive dialysis (48 h) against sodium phosphate buffer saline (pH=7.4). In the next parameters the effects of *M. nigra* L. and quercetin on fructose damaged DNA were investigated.

Fluorescence studies (360-500nm)

Flourescence spectra analysis of native DNA, fructosylated DNA, *M. nigra* L. and quercetin systems samples were conducted spectroflouro-photometer. The samples were excited at 360 nm and emission intensities were recorded in range of 380-500 nm. AGEs specific flourescence at 440 nm after excitation at 360 nm confirmed the prescence of possible AGEs in modified DNA samples[38]. Fluorescence intensities were calculated at the wavelength at which they were excited. Absorbance values and % AGEs inhibition values were calculated using the formula below.

$$\%F = [(A_{Control} - Sample) / A_{Control}] \times 100$$

UV-visible absorbance spectroscopy (200-400 nm)

The ultraviolet absorption profile of native and fructosylated DNA samples were recorded on UV-1700 Spectrophotometer in the wavelength range 200-400 nm using quartz cuvette of 1 cm path length[39]. Hyperchromicity was calculated at 260 nm.

$$\%Hyperchromicity = [(A_{Control} - Sample)/A_{Control}] \times 100$$

Fructosamine test (determination of amadori products in DNA)

Nitroblue tetrazolium test, changes to be caused by Amadori Products were detected in the dialyzed DNA samples. DNA sample (25 µg/mL) was incubated with solution containing 100 mM sodium carbonate buffer and (pH 10.8) 0.25 mM NBT at 37°C for 5 h. Amadori products formed NBT^+ radical by reducing NBT and purple color was observed. Then, the absorption values were read at 525 nm[7].

DNA oxidation

The oxidation effect of the OH radical formed as a result of photolysis of hydrogen peroxide with UV on DNA was examined in Agarose Gel Electrophoresis using pBR 322 plasmid DNA. The effects of *M. nigra* L. extracts and quercetin in the concentration range of 50-250 µg/mL to protect DNA against hydroxy radicals were investigated[36].

Samples were withdrawn from this mixture and mixed with 3 µL of loading buffer and loaded on 1% agarose gel. Gels were run at 60 V in tris-acetate-EDTA buffer until the dye came out of the gel. The gels were stained with ethidium bromide and the gel was scanned on gel documentation system[40].

Results and Discussion

AGEs can modifies protein and lipid groups. ROS formed during glycation disrupt the antioxidant system through AGEs. Thus oxidative-chronic stress it causes many diseases, especially diabetes and cancer.

Fructosylation of DNA results in the formation of DNA-AGEs. Fructosylation causes significant damage to DNA. This causes mutations such as DNA depurination, strand breakage, insertion and deletion. DNA-AGEs the loss of genomic integrity that occurs during aging can lead to cancer and other diseases. As a result of the literature review, there were not enough studies on the effect of *M. nigra* L. on DNA fructosylation. Then, it was decided to study the effect of *M. nigra* L. on the DNA-fructose system.

% Yield of plant extracts

In the study, the % yield was calculated for the *M. nigra* L. 96% ethanol-water extraction methods used (Table 1).

Total phenolic and flavonoid components

Phenolic substances are radical scavenging natural antioxidants. It is known that flavonoids inhibit lipid peroxidation and are radical scavengers[36]. The amount of phenolic component contained in *M. nigra* L. ethanol-water extracts used in the study was calculated as equivalent to gallic acid. The total flavonoid component assay was calculated as equivalent to Que (Table 2). Total phenolic component content was studied at 5 concentrations in the range of 10-90 µg/mL. The phenolic components contained in 1mg of *M. nigra* L. ethanol extracts; 76.3±0.003 µg GAE in the soxhlet method, 64.4±0.003 µg GAE in the maceration method, and 54.8±0.003 µg GAE in the ultrasound method were calculated. Total flavonoid component content was studied at 5 concentrations in the range of 50-250 µg/mL. The amount of flavonoids contained in 1 mg of *M. nigra* L. ethanol extract was calculated as 143.94±0.06 µg Que in the soxhlet method, 138.62±0.06 µg Que in the maceration method and 105.33±0.06 µg Que in the ultrasound method.

Total phenolic and flavonoid components in *M. nigra* L. ethanol extract; SE > ME > US. In similar studies, the amount of phenolic component in black mulberry fruit was calculated as GAE/g as equivalent to gallic acid (1422 mg/g), and the amount of flavonoid component was calculated as Que/g as equivalent to Que (276 mg/g)[24].

Compared with *M. nigra* L. fruit was determined that the amount of phenolic component was approximately 1/20 and the amount of flavonoid component was approximately 1/2. Polyphenolic compounds containing antioxidant substances are affected by some external physical factors such as light, climate, solvent[41].

DPPH radical scavenging activity

Antioxidant substances scaveng the DPPH radical by giving their own hydrogen to the radical. DPPH is a free and stable radical. The effect of scavenging DPPH radical is determined in a short time compared to other antioxidant methods. Therefore it is used more widely[34].

While determining the DPPH radical scavenging activity 6 concentrations of *M. nigra* L. ethanol extracts

Table 1 — Extractive yield (%) of different methods of *M. nigra* L.

Method	Herb Quantity(g)	Solvent Quantity (mL)	Extract Quantitiy (g)	% Yield
ME	20	500	8.52	42.6
SE	20	500	8.58	42.9
US	20	200	9.39	46.95

Table 2 —Determination of total phenolic and flavonoid components in *M. nigra* L.ethanol extract

Method	Fenolic component (µg GAE/mg extract)	Flavonoid component (µg Que/mg extract)
Maceration	64.4±0.003	138.62±0.06
Soxhlet	76.3±0.003	143.94±0.06
Ultrasound	54.8±0.003	105.33±0.06

were studied in the range of 10-500 µg/mL. *M. nigra* L. ethanol extracts showed scaveninging activity between 69.45% and 91.88%.

The maximum activity was 91.88% inhibition in the SE method 82.26% inhibition in the MEmethod 63.71% inhibition in the US method and BHT, which was the positive control showed 94.26% inhibition (Fig. 1.). The ethanol extract (SE method) of *M. nigra* L. was found to have thehighest antioxidant activity.

OH radical scavenging activity (deoxyribose assay)

Hydroxy radical scavenging activity was investigated in Fe^{2+}/ascorbate/EDTA/H_2O_2 system by deoxyribose method. After the hydroxy radical attacked deoxyribose, MDA (malondialdehyde) was formed as a result of sequential reactions. The resulting MDA reacted with TBA to form a pink colored MDA-TBA complex with absorbance at 532 nm. MDA can cross-link with DNA.

The OH radical scavenging activity of *M. nigra* L. ethanol extract obtained by the SE method was studied at 5 concentrations in the range of 5-25 µg/mL.

% Inhibitions; % I = $[(A_{Control} - Sample)/A_{Control}]$ x 100 was calculated with this equation.

BHA and BHT were used as positive controls. The hydroxy radical scavenging activity of metformin which is known to be antidiabetic and used in various treatments was also investigated. *M. nigra* L. ethanol extract showed hydroxy radical scavenging activity in the range of 33.33% to 64.97%. BHA if BHT is between 28.24% and 58.38% showed an inhibitory effect between 59.13% and 72.31%. metformin was determined that showed inhibition in the range of 9.03% to 43.31% (Fig. 2.).

Metformin is known to be a biguanide antidiabetic. At the same time shows antioxidant properties because it chelates metals such as iron and copper and destroys their toxic effects[42]. *M. nigra* L. ethanol extract showed higher activity than positive control BHA and showed almost the same inhibition as BHT. Metformin also had hydroxy radical scavenging activity.

Fluorescence studies (360-500 nm)

The effect of *M. nigra* L. and Que on AGE formation the excitation fluorescence at 360 nm and the emission fluorescence at 500 nm were measured and the fluorescence intensities at the excitation wavelengths were calculated[23].

%F= $[(A_{Control} - Sample)/A_{Control}]$ x 100

Morus nigra L. at concentrations of 50, 100, 150, 200, 250 µg/mL showed % inhibition in the range of 68% to 95% with increasing concentration. Que showed % inhibition in the range of 59.55% to 91.17% with increasing concentration (Fig. 3.).

The aldehydec apurinic/apyrimidinidic (AP) sites in DNA can be directly induced by reactive oxygen

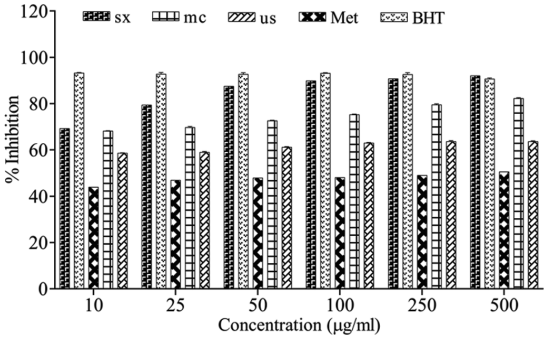

Fig. 1 —Scavenging effect of different concentrations (10, 25, 50, 100, 250, 500 µg/mL) of *M. nigra* L. ethanol extracts on the DPPH radical. Each value is given as the average of 3 test results and ± standard deviations (SD) (n=3)

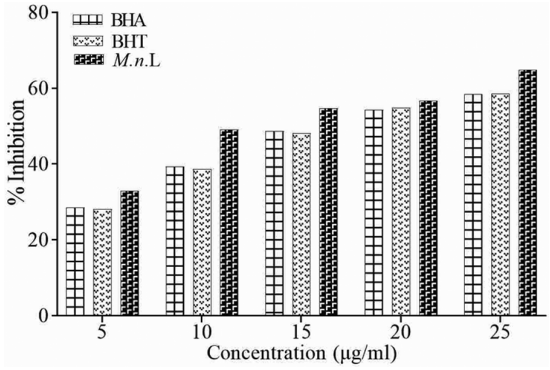

Fig. 2 — The hydroxy radical scavenging activity (%) of *M. nigra* L. ethanol extract of different concentrations (5, 10, 15, 20, 25 µg/mL). Each value is averaged over 3 test results and given ± standard deviations (SD) (n=3)

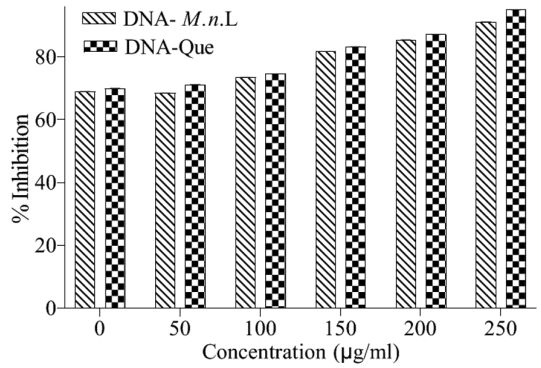

Fig. 3 — % Inhibition of DNA- *M. nigra* L. and DNA-Que at concentrations of DNA-Fructose system (50, 100, 150, 200, 250 µg/mL). Each value is given as the average of 3 test results and ± standard deviations (SD) (n=3)

species or result in removal of oxidized bases caused by glycosylase enzymes. This is detected by the increase in fluorescence intensity. The change in absorbance observed is due to changes in the molecular structure of the fructosylated DNA which may be due to the addition or substitution of DNA molecules mediated by the free radical.

Increased fluorescence intensity indicates the formation of DNA-AGE (CEdG)[43].

AGE formation in the DNA-fructose system was detected by the increase in fluorescence, and the inhibition of AGE formation by Que and *M. nigra* L. was detected by the decrease in fluorescence.

UV-visible absorbance spectroscopy (200-400 nm)

By using UV-1700 spectrophotometer, the effect of different concentrations of *M. nigra* L. and Que on natural, fructosylated DNA in a wavelength range of 200-400 nm and in a 1 cm long path were calculated by looking at the wavelength ranges absorbed by the samples, and their hyperchromicity at 260 nm[44].

$$\text{Hyperchromicity (\%H)} = [(A_{Control} - \text{Sample})/A_{Control}] \times 100$$

The absorption (absorption) of single-stranded DNA is higher than the absorption of double-stranded DNA this is known as a hyperchromic effect (meaning more colors). The hydrogen bonds between the doubled bases in the double helix limit the resonance behavior of the aromatic ring of the bases, resulting in a reduction in the UV absorbance of the double-stranded DNA (hypochromic effect). It forms hydrogen bonds with complementary bases resulting in high absorbance (hyperchromic).

Tight interactions between stacked bases in nucleic acid lead to a reduction in UV absorption compared to the same amount of free nucleotides. Absorption is also reduced when two strands are matched. This is called the hypochromic effect. The unwinding of the double-stranded nucleic acid also acts in the opposite direction. The increase in absorption is known as the hyperchromic effect. Therefore, the transition from the double helix to the single-stranded, denatured structure can be monitored by examining the UV absorption at 260 nm. Glycation of DNA which occurs in the form of nucleotide AGE is associated with increased mutation frequency and cytotoxicity[45] (Fig. 4.). The absorbance at 260 nm in DNA is due to the π-π interaction between the bases.

M. nigra L. SE extract (50, 100, 150, 200, 250 µg/mL) concentrations are showed hyperchromicity in the range of 19.724% to 16.255%. Same concentrations of Que are showed hyperchromicity between 18.505% and 8.002%.

Fructosamine test (determination of amadori products in DNA)

The process from DNA glycation to the formation of Amadori Products is reversible. Glycation is concentration dependent, more so in the early stages. Therefore, its production increases in diabetes[46-49]. Amadori products, which are formed in the early stages of DNA glycation, reduce NBT in alkaline medium and form a purple colored compound that absorbs at 525 nm. The NBT reduction test, which was developed for the measurement of fructosamine from early glycated products in DNA, was performed. AGE inhibition; with the formula calculated:

$$(\%I) = [(A_{Control} - \text{Sample})/A_{Control}] \times 100$$

The presence of superoxide was detected by the decrease of NBT. The increase in the absorbance of fructose DNA suggests the presence of superoxide radical[50-52]. The reason for the formation of superoxide is the glycation of DNA with fructose. It was thought that DNA-Fructose- *M. nigra* L. and quercetin inhibited the formation of fructosamine by inhibiting the superoxide radical. *M. nigra* L. SE extract (50, 100, 150, 200, 250 µg/mL) concentrations are showed inhibited fructose amine formation between 21.97% and 32.48%. Same concentrations of Que are showed inhibited fructose amine formation between 23.48% and 37.72% (Fig. 5).

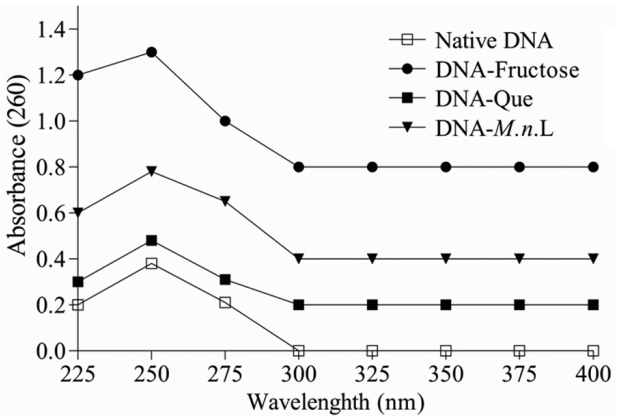

Fig. 4 — Ultraviolet absorption spectra of Native DNA, DNA-Fructose, DNA-Que, DNA- *M. nigra* L. (50, 100, 150, 200, 250 µg/mL) concentrations. Each value is given as the average of 3 test results and ± standard deviations (SD) (n=3)

Fructosylation of DNA

Plasmid DNA pBR 322 (0,25 µg/mL) was incubated at five different concentrations of fructose (10, 20, 30, 40, 50 mM) at 37°C for 5 days under steril conditions in 10 mM phosphate buffer saline (pH= 7.4) containing 0.01% sodium azide. After the specified time of incubation (5 days), unbound fructose was removed by extensive dialysis (48 h) against sodium phosphate buffer saline (pH=7.4). It was observed that Form I disappeared and Form II and Form III appeared as the fructose concentration increased. Fructose can cause mutations in biological macromolecules (such as strand breakage and depurination in DNA) through non-enzymatic reactions.As a result, DNA breaks were observed as the fructose concentration increased (Fig. 6).

The effect of *M. nigra* L. and quercetin on fructose-induced DNA damage

It was observed as Form I and more often as Form II. It was observed that Form III disappeared Form II and Form I were formed as the concentration of *M. nigra* L. SE increased (Fig. 7).

Form I was clearly observed as the Quercetin concentration increased (Fig. 8). For a system to be healthy it must be in a free radical/antioxidant balance. If this system is disrupted, biological macromolecule damage may ocur.ROS are one of the factors that increase the formation of AGEs.

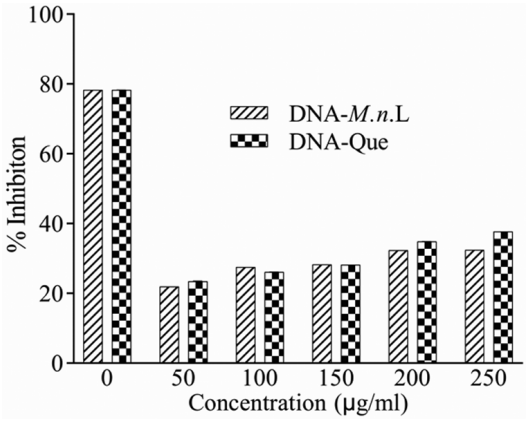

Fig. 5 — Spectroscopic examination of amadori products formed in the DNA-fructose system and inhibition of fructose amine formation by % inhibition of DNA- *M. nigra* L. and DNA-Que system (50, 100, 150, 200, 250 µg/mL). Each value is given as the average of 3 test results and ± standard deviations (SD) (n=3)

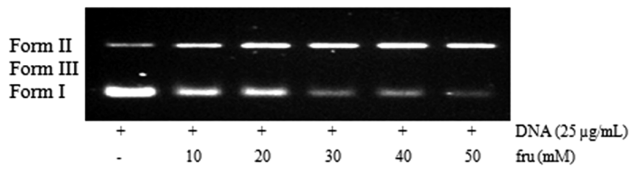

Fig. 6 —Fructoslytion of DNA with increasing concentrations of fructose (from Form I to Form II)

The effect of *M. nigra* L. and quercetin on H_2O_2 + UV induced DNA damage

Photolysis of H_2O_2 with UV was determined that the entire Form I was transformed into a Form II and Form III form. It was observed in the Gel documentation system that *M. nigra* L. was formed at 250 µg/mL concentration of SE where Form III was destroyed and Form I was formed. It was observed in the Gel documentation system that at all concentrations of Quercetin (Que) where Form III was destroyed and Form I and Form II were formed (Fig. 9). It was determined that *M. nigra* L. and quercetin (Que) inhibited the OH radical as an antioxidant and restored the supercoiled form (Form I) of DNA

Conclusion

The result of the study suggests that pBR322 DNA is highly susceptible to fructosylation and leads to

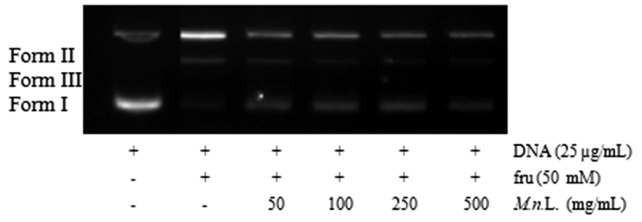

Fig. 7 — Effect of *M. nigra* L. ethanol extract on DNA fructosylation at increasing concentrations

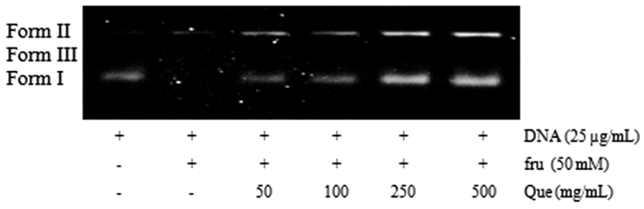

Fig. 8 — The effect of increasing concentrations of Que on DNA fructosylation

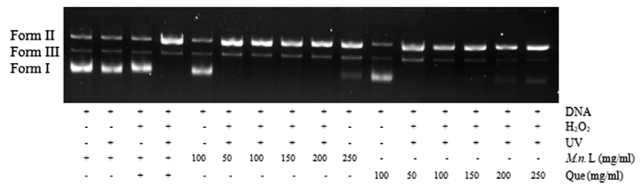

Fig. 9 —Agarose Gel Electrophoresis of the p*rot*ective effect of *M. nigra* L. ethanol extract and Que at different concentrations against the OH radical formed as a result of H_2O_2 photolysis of supercoiled

generation of fluorescent DNA-AGEs. A direct link between dietary component (consumption of starch-based sugar) and DNA fructosylation was observed. Fructose has been found to disrupt the structural integrity of DNA. *M. nigra* L. extract and quercetin were found to protect the structural integrity of DNA at increasing concentration. *M. nigra* L. and quercetin (Que), were determined to have the effect of scavenging the OH radical formed as a photolysis of H_2O_2 with UV. According to NBT, UV,and fluorescence tests, it prevents free radicals from damaging DNA during the initial phases of fructosylation by scavenging the radicals. ROS plays an important role in the formation mechanism of AGEs. If ROS is extinguished at an early stage the formation of AGEs is also prevented. Therefore the formation of diseases caused by AGEs is prevented. It has been determined as a result of experimental studies that *M. nigra* L. and quercetin (Que) extinguishes ROS. It was also found to prevent fructose-induced DNA damage. Studies have shown that *M. nigra* L. is effective as an AGEs inhibitor at an early stage. It seeks to ensure the continuity of this study for practical usage by supporting it with *in vivo* investigations.

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Conflict of interest

All authors declare no conflict of interest.

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