



ORIGINAL ARTICLE

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The protective effects of different parts of hypericum perforatum extracts on human mononuclear leukocytes in hydrogen peroxide-induced DNA damage and their phenolic contents

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Abstract

Oxidative stress is the state of the formation of some pathophysiological condition with the excessive increase of the normal amount of free radicals in the organism. In this study, the in vivo genotoxic and antigenotoxic effects of methanol and water extract and phenolic content of Hypericum perforatum flower, fruit, and seed methanol extracts were analyzed. HPLC was used to evaluate the quantities of 3,4-Dihydroxybenzoic acid, syringic acid, hydroxycinnamic acid, O-coumaric acid, caffeic acid, and catechin in the methanol extracts. The alkaline comet test was used to assess the DNA damage and protective effects of H. perforatum flower fruit, seed methanol, and water extract on human mononuclear leukocytes. The amounts of catechin and caffeic acid in seed methanol extract were found as quite high when compared to other extracts. The highest protective effects were seen at 10 and 50µg/ml concentrations of seed methanol extract. The optimum doses of fruit, flower, and seed extracts obtained from H. perforatum neutralized the genotoxic effect. This effect is stronger in seed methanol extract than other extracts. We suggest that more research is needed to evaluate the effects of H. perforatum phytochemicals in vitro and in vivo.

Keywords: DNA damage, H. perforatum, Catechin, Caffeic Acid, 3,4-Dihydroxybenzoic

Introduction

The oxidant-antioxidant system of a living organism is in balance and this is necessary for a healthy life. Free radicals are produced endogenously during the normal metabolic process in life. In addition, external factors such as sun rays, radiation, smoking, and environmental pollution also cause free radical formation. Free radicals, because of their reactive nature, have the potential to interact with and damage all cell components, particularly lipids, nucleic acids, and proteins [1]. Oxidative stress has been frequently accepted as a related factor in aging, health maintenance, wellness, and prevention of chronic and degenerative diseases [2,3].

Some secondary metabolites, such as phenolic acids, flavonoids, steroids, alkaloids are important; they play an important role in preventing disease. These compounds are significant, antioxidant properties and contribution to health. Phenols, which are abundant in medical plants, attract great interest from researchers [4]. Hypericum species are included in the Clusiaceae families and subfamilies Hypericaceae include approximately 400 species in the world and is known as St John's wort. Hypericums are used in traditional medicine all around the world. Hypericum species have very valuable phytochemical properties. That's why Hypericum species are widely used in traditional medicine for the treatment of depression, type II diabetes, wound healing, muscle aches, and treatment of burns [5,6]. Hypericum perforatum contains hyperforin, hypericin, quercetin, epicatechin, catechin, tannins, resveratrol, biapigenin, porphyrins, flavonoids, flavonoid derivatives, and xanthone derivatives. As we know, these compounds exhibit antioxidant activity, inhibition of lipid peroxidation, and free radical scavenging properties [7]. Many clinical trials have shown that consuming nutritional supplements,

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protective foods, phytochemicals, and modified diets regularly can help prevent damaged cells and many cancer types [5,8]. The purpose of this study is to see if methanol and aqua plant extracts from *Hypericum perforatum* can protect human mononuclear leukocytes from oxidative stress and DNA damage caused by H₂O₂ using the single-cell alkaline gel electrophoresis method.

Materials and Methods

Preparation of methanol and aqueous extracts

H. perforatum specimens From May to June, were collected from the countryside of Zinnar Village (Mardin) during the flowering, fruiting, and seeding stages. Plant materials weighing 100 g were dried. A total of 30 g of flower, fruit, and seed samples were ground in a grinder with a 2 mm diameter mesh and incubated for 3 days at room temperature with 200 mL (99 percent) of methanol. The solvent phase was removed using a rotary evaporator under a low vacuum. Each part yielded approximately 3 g of crude methanol extract. Decoctions of aqueous plant extracts were made by combining 30 g of ground dry plant material with 200 ml of water.

Instrumentation

HPLC (Agilent 1260 Infinity) was used to determine the phenolic content of *H. perforatum* crude methanol extracts. The Alkaline Comet Assay was performed using a centrifuge (Hettich Universal 30 RF), microscope (Olympus CKX41), and a Labotect CO₂-incubator.

Chromatographic analyses

HPLC was used to examine the phenolic components in methanol extracts of various sections of *H. perforatum*. The HPLC (C18 column 250x4.6–5mm; reversed-phase) set at 35°C was used to separate phenolic chemicals. For gradient elution, methanol and acetic acid-water (2:98 v/v) were utilized. 10mg of fruits, flowers, and seeds crude extract were dissolved in 10ml of methanol and filtered with a microfilter (millipore 22µm) before being injected into HPLC. At a wavelength of 280 nm, the phenolic chemicals were detected.

Comet Assay Method

DNA, which carries genetic information and transmits it from generation to generation, is an easily damaged molecule and is constantly damaged. Damage is a change in the chemical and physical structure of normal DNA. Damage in DNA (deoxyribose phosphate skeleton), typical alteration of purine and pyrimidine bases, and DNA-protein cross-linking are all caused by free radicals. The "Single Cell Gel Electrophoresis" (SCGE) technology, which has only recently been developed, is a sensitive, quick, and low-cost method for detecting DNA helix breaks. "Comet Assay" or "Microgel Electrophoretic Technique" is another name for the SCGE technique. Singh et al. used an alkaline comet test to examine DNA damage in mononuclear leukocytes [9,10].

The Microgel Electrophoretic Technique, also referred to as Sister Chromatid Exchange (SCE) and Microgel Electrophoresis (MGE), is the most commonly used test for human cells to investigate DNA damage. With these methods, the detection of DNA strand breaks can be accomplished precisely, quickly, and safely. In genotoxic

screening, the method comet is gradually gaining more acceptance. The Comet method has many applications in the field of toxicology, from aging to genetic toxicology and molecular epidemiology. In this study, the alkaline single-cell electrophoresis method (Comet Assay) was preferred in determining anti-genotoxic activity.

Anti-genotoxic activity

Human mononuclear leukocyte separation and incubation

A 28-year-old healthy, non-smoking male consented to donate a 20-ml heparinized blood sample. The tubes were carefully filled with 5 mL of Histopaque-1077 and heparinized blood. For blood cells, samples were centrifuged (3.103 rpm; 30 min). After the separation the lymphocytes were removed into other tubes. 5 mL PBS was transferred into each test tube to remove the Histopaque solution. The test tubes were centrifuged (3.103 rpm) for 10 minutes. Finally, the upper layer of centrifuged fluid was taken with a micropipette and the mononuclear leukocyte cells were separated [11].

Cell viability test

To evaluate the viability of mononuclear leukocyte cells trypan blue (TB) stain was employed. The cells in the culture flask were trypsinized and removed. An equal volume (1:1) of TB was added to the cells. For five minutes, they were incubated. At the end of the incubation period, stained and unstained cells were counted using a microscope [12].

Cell suspension cultures

Preparing of human mononuclear leukocyte cells culture Dulbecco's modified Eagle's medium (DMEM) was used. DMEM was supplemented with 10% FBS and 5 g/mL gentamicin antibiotic. The cell cultures were incubated in a CO₂ incubator (5 percent CO₂, 95 percent humidity, and 37°C) for 1, 2, and 3 hours. Leukocyte cells without being exposed to hydrogen peroxide and extracts were used as a negative control group. To induce cells damage 0.7 mM H₂O₂ was used as a positive control. Mononuclear leukocytes were treated with *H. perforatum* methanol extracts obtained from different parts at various concentrations (10-100µg/ml).

Investigation of DNA damage with Alkaline Comet Assay

DNA damage, induced with H₂O₂ and the protective effect of obtained extracts on human mononuclear leukocytes were investigated by the alkaline comet assay method. [10]. Briefly, 80 µl of 0.7% agarose (low melting point) was added in PBS and cooled to 37°C. Then PBS was mixed with 10 µL (2x10³ cells) of cell culture suspension which was treated with plant extract (10-100µg/ml).

Results

Our study aims to determine to phenolic content of *H. perforatum* flowers, fruits, and seeds methanol extract and investigate genotoxic and antigenotoxic effects on H₂O₂ induced DNA damage. Phenolic acids are secondary metabolism products of plants and are synthesized in response to biological and physical stress factors. In addition, they can show many medicinal properties such as antioxidants, anticarcinogenic, antidiabetic,

antidegenerative, and antithrombotic for human health [13]. For these reasons, polyphenolic compounds have been potential biomolecules for the prevention or treatment of various diseases, especially neurodegenerative diseases, cardiovascular diseases, cancer, and premature aging [14,15]. These phenolic compounds are phytochemicals that are naturally found in fruits, vegetables, grains, and various plant products and are responsible for various characteristic features of these foods, such as color, taste, and smell. These biologically active phytochemicals consist of a large group of compounds containing aromatic benzene rings with one or more hydroxyl (-OH) groups attached, derived from the shikimic acid pathway and phenylpropanoid metabolism [16]. These functional groups are associated with antioxidative metabolism. Moreover, they serve as a defense mechanism against a variety of pests, including viruses and parasites [17]. Various studies have shown that phenolic compounds have antioxidant, anti-inflammatory, antidiabetic, anticancer, antimicrobial, neuroprotective, cardioprotective properties [18-20].

The amounts of caffeic acid, protocatechuic acid (PCA), hydroxycinnamic acid, catechin, syringic acid, and o-coumaric acid in crude extracts were determined by using HPLC. Figure 1 shows a typical combination of phenolic chemicals and associated retention time chromatograms.

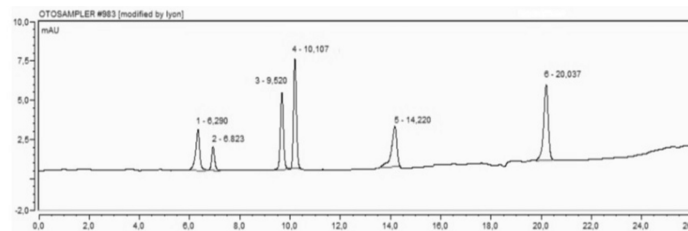


Figure 1. A typical chromatogram of a conventional phenolic compound combination. 1: 3,4-Dihydroxybenzoic acid (Rtime: 6.290); 2: Catechin (Rtime: 6.823); 3: Caffeic acid (Rtime: 9.520); 4: Syringic (Rtime: 10.107); 5: Hydroxycinnamic acid (Rtime: 14.220); 6: o-coumaric acid (Rtime: 20.037).

It was determined that these components were found in different amounts in methanol extracts obtained from different parts (flower, fruit, and seed) of the plant. Only the flower (12.93 mg/kg) and fruit (6.72 mg/kg) extract contained 3,4-Dihydroxybenzoic acid (PCA) resemble other antioxidants such as gallic acid, caffeic acid, vanillic acid, and syringic acid in structure. Catechin (flavan-3-ol) was found in 2.91, 119.14, and 13.45 mg/kg in flower, fruit, and seed extracts, respectively. Caffeic acid was also only found in the methanol extract of the fruit (12.97 mg/kg) and the seed (57.13 mg/kg) (Table 1). In extract samples, syringic acid, o-coumaric acid, and hydroxycinnamic acid were not present in measurable amounts.

Table 1. Phenolic compounds (mg/kg) of *Hypericum perforatum* methanol extracts

	3,4-Dihydroxybenzoic acid	Catechin (flavan-3-ol)	Caffeic Acid	Syringic Acid	Hydroxycinnamic acid	O-qumaric Acid
Flower	12.93	--	2.91	--	--	--
Fruit	6.72	13.45	12.97	--	--	--
Seed	ND	119.14	57.13	--	--	--

Caffeic acid has anti-viral and anti-tumoral, anti-inflammatory, antiproliferative, immunomodulatory properties with important antioxidant phenolic compounds. This extraordinary compound has been used to prohibit oxidative stress-based breakdown in cells [21,22].

Discussion

Catechins (flavan-3-ol) are generally detected in the seeds and skins of fruits. They have a structure containing two benzene rings and a dihydropyran ring. Catechins have strong antioxidant and anti-cancer effects due to the charms in the A and B rings. Protective effects against various oxidative damages have been demonstrated by examining the amounts of non-enzymatic and enzymatic antioxidants together with protein oxidation, lipid peroxidation, and superoxide radical measurements in the liver [23,24]. We detected the amount of catechin in the fruit and seeds of the plant. It was especially high in the seeds (119.14 ng/ml). Caffeic acid and its derivatives are synthesized against plant pathogens, environmental high and low-temperature stress, harmful rays, and biological and physical stress conditions of the plant such as drought, thirst, heavy metal stress, and salinity stress [25]. Caffeic acid (flavan-3-ol) has shown strong antioxidant activity, increased collagen production, and prevention of premature aging, as well as antimicrobial activity, and is promising in the treatment of various skin diseases [26]. It has been shown in some in vitro and in vivo

studies that caffeic acid has a protective and healing role against various cell and DNA damages [27]. Hydroxycinnamic acid (PCA) is a type of phenolic acid that is widely found in nature. PCA is among the best-known antioxidant compounds with structural similarities to syringic acid, caffeic acid, vanillic acid, and gallic acid. In vitro and in vivo, PCA has been demonstrated to be useful for the treatment and/or prevention of a wide range of illnesses linked to oxidative stress damage in many bodily systems [28]. Studies have shown many effects of PCA, such as anticancer, antiulcer, antidiabetic, antimicrobial, antioxidative, antithrombotic, and antiatherosclerotic activities [29]. The strong antigenotoxic properties of *H. perforatum* extracts (flower, fruit, and seed) were determined using mononuclear leukocyte cells injured by HO_2O_2 (Table 2).

The microgel electrophoretic technique was used to assess mononuclear leukocyte DNA damage levels after 1, 2, and 3 hours in the prepared cell culture flask. The density of fluorescence in the comet tail was evaluated as undamaged (0) minimum or maximal damage (1 to 4), resulting in a total score for each slide ranging between 0 and 400 arbitrary units (AU). Figures 2 a and b show photomicrographs of typical samples. The strongest antigenotoxic effects were seen at the flower and seed methanol extract concentrations of 10 and 50g/ml, respectively. Anti-genotoxic effects were reduced at doses below and above these values. Table 2 shows the anti-genotoxic effects of all applied concentrations of *H. perforatum* flower, fruit, and seed methanol and water extracts,

as well as positive/negative controls. As can be seen in Table 2, a strong antigenotoxic effect was determined with the addition of 50 µg/mL of the seed extract of the *Hypericum perforatum* plant obtained with methanol solvent, and a good antigenotoxic effect

was determined with the addition of 10 µg/mL. Likewise, a strong antigenotoxic effect was detected with the addition of 50 µg/mL extract of the flower extract obtained from the methanol extract of the *H. perforatum* plant and the addition of 25 µg/mL extract.

Table 2. Antigenotoxic effect of *H. perforatum* methanol extracts induced by H₂O₂ in human mononuclear leukocytes

Exposed Concentration	Hypericum perforatum methanol extracts Arbitrary Unit (AU)		
	Flower	Fruit	Seed
10 µg/mL extract and 0.7 mM H ₂ O ₂	183.24±2.04	221.65±3.50	86.41±5.54
25 µg/mL extract and 0.7 mM H ₂ O ₂	116.52±5.37	136.28±12.20	159.31±3.77
50 µg/mL extract and 0.7 mM H ₂ O ₂	83.70±5.51	258.57±4.12	47.24±2.04
100 µg/mL extract and 0.7 mM H ₂ O ₂	218.33±6.50	297.12±12.21	113.03±6.96
Control	26.33±5.85	23.15±1.03	22.57±4.50
Positive control 0.7 mM H ₂ O ₂	271.33±6.42	321.33±11.01	272.66±4.16

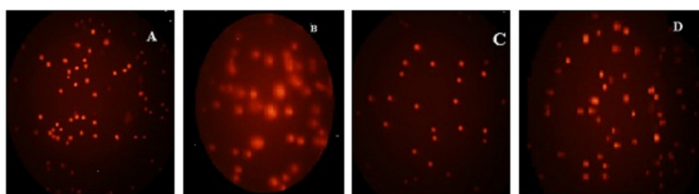


Figure 2a: DNA damage visual classification, according to the relative proportion of DNA in the tail (cells between 0-4), provided from single-cell gel electrophoresis. Undamaged cell (0), and the most heavily damaged cell (4). (a) Control cells, (b) Treated only with 0.7 mM H₂O₂ (Positive control), (c) 25 µg/ml HPFE + Positive control, (d) 50 µg/ml HPFE + Positive control, HPFE: *H. perforatum* flower extract; Positive control: H₂O₂ (Hydrogen peroxide)

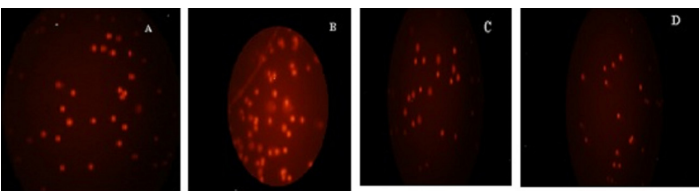


Figure 2b: DNA damage visual classification, according to the relative proportion of DNA in the tail (cells between 0-4), provided from single-cell gel electrophoresis. Undamaged cell (0), and the most heavily damaged cell (4). (a) Control cells, (b) Treated only with 0.7 mM H₂O₂ (Positive control), (c) 10 µg/ml HPSE + Positive control, (d) 50 µg/ml HPSE + Positive control, HPSE: *Hypericum perforatum* seed extract; Positive control: H₂O₂ (Hydrogen peroxide)

Conclusion

H. perforatum is one of the most commonly used herbs in ancient folk medicine. It has been used for centuries in the treatment of diseases in our country as well as all over the world. The use of phenolic compounds and their extracts from medicinal plants has revived and accelerated the development of these products in foods and various disease prevention and treatment regimens [30]. The wound-healing properties of this plant have been known and used for centuries. Recently, its antibacterial, antiviral, and antifungal properties have also been demonstrated. Alcoholic extracts of *Hypericum* species have proven to be more active than aqueous solutions. [31]. It is possible to say that extracts obtained with methanol from the seed, flower, and fruit of *H. perforatum* show strong anti-genotoxic activity against H₂O₂ treated human mononuclear leukocytes. The biological activity of the extracts was

validated by HPLC measurement of protocatechuic acid, catechin, and syringic acid. According to the findings of our research, *H. perforatum* may be an excellent and favoured plant as an antioxidant source. Optimal dosages of *H. perforatum* seed, flower, and fruit extracts exhibit a substantial anti-genotoxic activity, which is greater in seed extracts than in fruit and flower extracts. In general, *H. perforatum* is thought to be an excellent source of antioxidants. It implies that it might be a source of potentially helpful substances in many oxidative damage scenarios. In particular, alcohol-based extracts and various bioactive components of this plant may be beneficial for public health and the pharmaceutical industry. More work is needed on it.

Conflict of interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

The authors declare that they have received no financial support for the study.

Ethical approval

Our study does not require ethics committee approval.

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