The Genoprotective Effects of Plantago lanceolata Extract and Luteolin-7-B-D-Glycoside on Lymphocyte and HEPG-2 Cells

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Abstract: In this study, it was aimed to investigate the possible genoprotective effects of Plantago lanceolata (Plantago L.) plant extract, which has been various phytotherapeutic properties, especially wound healing, and Luteolin-7-B-D-glicoside (LUT-7G), one of the important components of the plant, on human lymphocytes and HEPG-2 cells.

The genoprotective effects of Plantago L. extract (200 and 500 μg/ml) and LUT-7G (10, 20, 30 μM) on lymphocytes and HepG-2 cells with and without DNA damage were investigated using comet analysis.

In the study, while DNA damage (% DNAγ) increased only in the high concentration groups of plant extract and LUT-7G in lymphocyte cells, it increased in all groups in HEPG-2 cells. In the DNA damaged cells, comet parameters decreased at all concentrations in lymphocytes however it decreased at only plant groups and 10 μM LUT-7G group in HEPG-2 cells.

Plantago L. extract and LUT-7G showed genotoxic effect at all concentrations in HEPG-2 cells and only in high concentrations in lymphocytes but test substances showed genoprotective properties by reducing genetically damaged cells. Thus, we can say that the Plantago L. and LUT-7G are antigenotoxic in healthy cells, and genotoxic in cancer cells, depending on the dose.

Keywords: Plantago L., Luteolin-7-B-D-glicoside, genotoxicity, genoprotective, HEPG-2, lymphocyte

1. INTRODUCTION

Plantago is a perennial plant genus with a wide distribution in the world and there are 21 species in Turkey. Plantago major and Plantago lanceolata (Plantago L.) are the most common types (Figure 1a). It is popularly known as nerve grass, vein grass, edgy leaf, Turkey. Plantago major and Plantago lanceolata ([1]

The medicinal properties of plants are due to their ability to produce pharmacologically active compounds. Active compounds in plants are secondary metabolites defined as phytochemicals and flavonoids constitute an important group of secondary metabolites. Flavonoids are known to have roles in biotransformation, preventing low-density lipoprotein oxidation, and destroying free radicals [4]. Plantago L.; It is rich in flavonoids (baisalein, luteolin), monoterpenoids (linalool), triterpenoids (oleanolic acid, urcholic acid), iridoidglycosides (aucubin) and phenolic components. Glycoside and phenol, which are components of Plantago L., give the plant anti-inflammatory properties. Luteolin-7-B-D-glycoside (LUT-7G) is one of the most important components of the Plantago L. plant and is a potent anticarcinogenic agent that can stop the proliferation of various cancer cells [1, 5]. LUT-7G has also been reported to have antimutagenic, anti-inflammatory, anti-oxidant and antiproliferative activities [6].

Genotoxicity; It is a general concept that includes deformations such as DNA inserts, DNA breaks, gene mutations, chromosome anomalies, clastogenicity and aneuploidy formed in nucleus, chromosome and DNA structure. The comet technique, which is one of the tests that enables the relationship between the genetic systems and the carcinogenic and mutagenic potentials of the substances whose genotoxicity is to be tested, is a sensitive, fast and reliable method used for the detection of DNA single and double strand breaks caused by various agents.
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In this study, the possible antimutagenic effects of the methanolic extracts obtained from the above-ground plant parts of the Plantago L. plant and LUT-7G, one of the main components of the plant, were reported on HEPG-2 cells from hepatocarcinoma cells in order to show its effects on cancer cells as well as healthy lymphocyte cells. It was investigated together with comet test.

2. MATERIAL AND METHOD

2.1. Chemicals

LUT7-G, EtBr, HMA, LMA, DMEM and Trypsin-EDTA (Sigma); DMSO (PanReac AppliChem); Chromosome Medium P (EuroClone); Histopaq 1077, HEPES, FBS, DMSO (Merck). All other chemicals used were of analytical grade. All materials were used as received without further purification. All aqueous solutions were prepared with ultrapure Milli Q (MQ) grade water.

2.2. Preparation of Test Materials:

Plant material and extraction: The Plantago L. was collected from natural populations around Malatya-Turkey in May-June 2019. It was defined by the Faculty of Pharmacy of İnönü University and registered in the herbarium (Figure 1b: Plantago L.8057-2019). The above ground parts of the collected plant material were dried at room temperature. 10 g of dry plant parts were dissolved in 100 ml of methanol solution, which had the least carcinogenic effect, at a rate of 1/10 overnight. The process was repeated three times. After filtering the mixture with filter paper (Whatman no. 1), the plant extract was prepared by removing methanol under vacuum using a rotary evaporator. The extracts were stored in sterile bottles.

LUT-7G Preparation: LUT-7G was dissolved in a DMSO ratio of at most 1%.

2.3. Experiment Protocol:

Lymphocyte Isolation and Cell Culture:

Approximately 3 ml of blood samples were collected from two healthy donors (one female and one male, 24-26 years old) who did not smoke, have no chronic disease, and had not been exposed to genotoxic agents recently and transferred in sterile heparin tubes. Lymphocytes were isolated by histopaq-1077 density gradient centrifugation method. Lymphocytes were washed with PBS buffer. It was resuspended in cold PBS at 5100-1100 cells/ml. Cell viability was determined in trypan blue. Cells with a viability rate above 90% were plated into 6 well plates at 106 cells.

HEPG-2 Cell Culture:

HEPG-2 cells, a human hepatocellular cancer cell line, were used in the study. HEPG-2 cells stored at -80 °C were kept at -20 °C for 1 night and then at + 4 °C for a short time, and then removed from the medium by centrifugation. After adding 5 ml of new medium to the cells, it was incubated at 37 °C and 5% CO2 atm for 2 days. When the cells formed sufficient colonies, the medium was removed and the cells were allowed to rise from the flask surface with Trypsin-EDTA. After centrifugation at 1000 rpm for 5 minutes, the supernatant was removed. In this way, the cells were washed twice. One-fourth of the cells obtained were evenly distributed in 6 well plates and 3 ml of DMEM was added to complete and incubated for 2 days. Cell viability was determined using 0.4% trypan blue and experiments were started when the viability rate was above 90%.

Experimental Groups and Study Doses:

Twelve experimental groups were formed separately for both cell types in the study. In addition to the control group in which no test substance was applied, a positive control group was formed, in which H2O2, which is known to cause DNA damage with an application dose of 200 μM, was applied. Two different concentrations (200, 500 μg / ml) for plant extract Harutyunyan et al. was applied to both cell types referring to studies [7]. On the other hand, LUT-7G was applied to the cells by selecting the concentrations of 10, 20, 30 μM applied in the study conducted by Zang et al. [8]. Again, the same concentrations of test substances were applied in combination after DNA damage was induced with 200 μM H2O2 in both lymphocytes and HEPG-2 cells. Cells were exposed to test substances for 24 hours.

Comet Test

The alkaline comet assay was performed using an adaptation of the method of Singh et al. [9]. Fully frosted microscope slides were dipped briefly into 0.7% hot (60C) normal melting agarose (NMA)
prepared in PBS. The slides were dried overnight at room temperature and then stored at 4°C until usage. Aliquots of 15 μl lymphocyte cell suspension (about 50,000 cells) were mixed with 120 μl low melting point agarose (LMA) (0.5% in PBS) and added to microscope slides (with frosted ends), which had been covered with a bottom layer of 1.5% LMA. The slides were maintained on an ice-cold flat tray for 15 min to solidify. Slides were then carefully immersed in cold lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH 10) with 1% Triton X-100 and 10% DMSO added just before use, for at least 1 hour at 4°C.

The slides were removed from the lysing solution, drained and placed in a horizontal electrophoresis tank. The tank was filled with fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) to a level just covering the slides. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of the DNA and the expression of alkali-labile damage. Subsequently, the DNA was electrophoresed for 30 min at 300 mA and 15 V. To prevent additional DNA damage, all steps described above were conducted in the dark and at 4°C. After electrophoresis, the slides were taken from the tank and washed three times (5 min each) with 0.4 M Tris buffer, pH 7.5 to neutralize the excess alkali.

To each slide, 50 ml ethidium bromide (EtBr – 20 mL/mL) was added. The slides were covered with a cover-slip, stored in a humidified box at 4°C and analyzed using a fluorescence microscope within 3–4 hours.

Cells per subject were analyzed at 40 magnification, under a fluorescent microscope (Olympus, BX51) equipped with an excitation filter of 546 nm and a barrier filter of 590 nm. Comets are formed upon the principle of releasing damaged DNA from the core of the nucleus during electrophoresis. Percentage DNA in tail (DNA\textsubscript{T}) on 100 cells per sample (two duplicate sample slides, 50 randomly selected cells scored per slide) was scored using image analysis software (BAB Bs200Pro Image Processing and Analysis System of BAB Muh., Turkey). Analysis was performed blindly by one slide reader.

The total comet DNA (DNA\textsubscript{C}) is the sum of the intensities located in the comet area. Head DNA (DNA\textsubscript{H}) is the sum of the intensities located in the head area. DNA\textsubscript{T} is the difference between total comet DNA and head DNA (DNA\textsubscript{T} = DNA\textsubscript{C} – DNA\textsubscript{H}). Percent tail DNA (%DNA\textsubscript{T}) is calculated as 100 x DNA\textsubscript{T} / (DNA\textsubscript{T} + DNA\textsubscript{H}). These results were expressed as mean percentage tail DNA (%DNA\textsubscript{T}) + SD [10].

2.4. Statistical Analysis:

The compatibility of the data of comet parameters obtained as a result of microscopic examination to normal distribution was examined by Kolmogorov-Smirnov test. Data were summarized with median, minimum, and maximum values. In group comparisons, Kruskal-Wallis variance analysis and finally Conover pairwise comparison method were used. Statistical evaluations were made using Windows for SPSS Version 16.0 software program. The significance level was accepted as P <0.05 in all tests.

3. RESULTS

Effects of Plantago L. extract and LUT-7G on Comet Parameters in Lymphocyte Cells:

Comet data obtained 24 hours after applying different concentrations of Plantago L. extract and LUT-7G to lymphocyte cells with and without DNA damage with H\textsubscript{2}O\textsubscript{2} were compared with the control groups (Table 1). Accordingly, the % DNA\textsubscript{T} values were 28.50 in the control group and 49.91 in the positive control group. Thus, it was revealed that H\textsubscript{2}O\textsubscript{2} exposure significantly increased DNA damage. While application of 200 μg/ml plant extract caused a decrease in comet parameters, it was determined that 500 μg/ml concentration significantly increased comet values. It was determined that LUT-7G was genotoxic only at high concentration (30 μM) and other doses were not significantly different from control group.

In our study, different doses of plant extract and LUT-7G were also applied to lymphocyte cells with DNA damage with H\textsubscript{2}O\textsubscript{2}. In these groups, both doses of plant extract showed antigenotoxic effect by decreasing the comet parameters statistically significantly compared to positive control. Among the groups, the antigenotoxic effect of low-dose plant extract application was found to be more significant than that of the high-dose group. Similarly, in the lymphocyte cells with DNA damage, the mean %
DNA_T in LUT-7G groups were found to be significantly lower than the positive control value. This antigenotoxic effect was more pronounced in the 20 μM administration group.

**Effects of Plantago L. extract and LUT-7G on Comet Parameters in HEPG-2 Cells:**

Table 2 shows the comet analysis data as a result of the treatment of different doses of *Plantago L.* methanol extract and LUT-7G in HEPG-2 cells. According to this; It was determined that mean % DNA_T of Plantago L. increase d significantly in all concentrations compared to control. The genotoxic effect of the high dose of plant extract was more than the low dose. A significant increase in comet parameters was detected in all doses of LUT-7G compared to the control and while the 20 and 30 μM doses of LUT-7G did not differ between the groups, the increase in the 10 μM dose group was higher than the others.

In HEPG-2 cells damaged by H_2O_2, the ratio of DNA damaged cells was significantly decreased in all groups treated with *Plantago L.* extract. On the other hand, only the low dose group of LUT-7G significantly decreased the proportion of DNA damaged cells.

**Figure 1a: Plantago lanceolata** and **1b: Inonu Univ. herbarium No: 8057-2019**

<table>
<thead>
<tr>
<th>Table 1. Comparison of comet damage scores in lymphocytes treated with different doses of *P.*lanceolata extract and LUT-7G for 24 hours.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Groups (n=50)</strong></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>H_2O_2 (Pozitive control)</td>
</tr>
<tr>
<td><em>P. Lanceolata</em></td>
</tr>
<tr>
<td></td>
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<tr>
<td>LUT-7G</td>
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<tr>
<td>LUT-7G</td>
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<tr>
<td>LUT-7G</td>
</tr>
<tr>
<td><em>Plantago L.+ H_2O_2</em></td>
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<tr>
<td><em>Plantago L.+ H_2O_2</em></td>
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<tr>
<td>LUT-7G + H_2O_2</td>
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<tr>
<td>LUT-7G + H_2O_2</td>
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<tr>
<td>LUT-7G +H_2O_2</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
</tr>
</tbody>
</table>

**NOTE:** Upper symbols differ significantly between control groups, within and between groups.

Table 2. Comparison of comet damage scores in HEPG-2 cells treated with different doses of *P.*lanceolata extract and LUT-7G for 24 hours.
The Genoprotective Effects of Plantago lanceolata Extract and Luteolin-7-B-D-Glycoside on Lymphocyte and HEPG-2 Cells

<table>
<thead>
<tr>
<th>Groups (n=50)</th>
<th>Group Symbol</th>
<th>Concentration</th>
<th>DNA tail Median (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>A</td>
<td>-</td>
<td>28(18.92-40.02)</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>B</td>
<td>200 µM</td>
<td>49.74(27.05-69.25)</td>
</tr>
<tr>
<td>Plantago L.</td>
<td>C</td>
<td>200 µg/ml</td>
<td>41.18(29.6-52.28)</td>
</tr>
<tr>
<td>Plantago L.</td>
<td>D</td>
<td>500 µg/ml</td>
<td>44.29(34.23-55.52)</td>
</tr>
<tr>
<td>LUT-7G</td>
<td>E</td>
<td>10 µM</td>
<td>53.91(41.48-66.95)</td>
</tr>
<tr>
<td>LUT-7G</td>
<td>F</td>
<td>20 µM</td>
<td>50.29(39.22-60.04)</td>
</tr>
<tr>
<td>LUT-7G</td>
<td>G</td>
<td>30 µM</td>
<td>50.26(37.3-61.86)</td>
</tr>
<tr>
<td>Plantago L.+H$_2$O$_2$</td>
<td>H</td>
<td>200 µg/ml</td>
<td>42.13(25.49-69.33)</td>
</tr>
<tr>
<td>Plantago L.+H$_2$O$_2$</td>
<td>I</td>
<td>500 µg/ml</td>
<td>42.37(28.93-60.67)</td>
</tr>
<tr>
<td>LUT-7G+ H$_2$O$_2$</td>
<td>J</td>
<td>10 µM</td>
<td>45.28(30.71-62.83)</td>
</tr>
<tr>
<td>LUT-7G+ H$_2$O$_2$</td>
<td>K</td>
<td>20 µM</td>
<td>50.01(30.05-71.06)</td>
</tr>
<tr>
<td>LUT-7G+ H$_2$O$_2$</td>
<td>L</td>
<td>30 µM</td>
<td>51.46(32.77-72.56)</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

NOTE: Upper symbols differ significantly between control groups, within and between groups.

4. DISCUSSION

Identifying plants with antimutagenic properties has recently gained importance in terms of preventing mutations that play an important role in the development of various chronic and degenerative diseases, including cancer [11]. In this respect, it is important to identify the ingredients with potential antimutagenic properties and to isolate the plant extracts. Bio-antimutagenes are generally effective intracellular agents and affect the process after mutagens interact with DNA. Antimutagenes can act directly, such as chemical or enzymatic inactivation of mutagens, as well as act as regulators of DNA replication and repair by binding to DNA. They also act indirectly by scavenging free radicals produced by mutagens [12-14].

This study was planned to reveal the genoprotective properties of Plantago L., which is consumed by the public for various therapeutic purposes, especially for its wound healing properties, in DNA damaged cells. Considering the inadequacy of the cumulative effects of plant extract components in explaining the molecular mechanism, different doses of LUT-7G flavonoid, which is the main component of the plant, were also included in the study. Studies on the cytotoxic/antitoxitoxic and genotoxic/antigenotoxic effects of Plantago L. and LUT-7G together are limited.

In our study, H$_2$O$_2$ was used as a positive control for the comet test. H$_2$O$_2$ Fenton, which is used to induce DNA damage, causes the formation of many oxidants such as hydroxyl radical and hypochlorous acid by playing a role in the formation of oxidized base by Haber-Weiss reactions. It also leads to damage in DNA such as single or double strand breaks and base modifications induced by ROS [15]. In our study, it was seen that comet formation increased significantly with H$_2$O$_2$ administration in both cell types, thus demonstrating the high genotoxic potential of H$_2$O$_2$.

In our study, it was determined that only high concentration (500 µg/ml) of Plantago L. extract significantly increased comet values in human lymphocyte cells. In lymphocyte cells genetically damaged by H$_2$O$_2$, it was determined that both doses of the plant extract significantly decreased the frequency of DNA damaged cells and had an antigenotoxic effect. In addition, the antigenotoxic healing effect of low-dose plant extract application was found to be more significant than that of the high-dose group. Although the antioxidant, antigenotoxic and anticarcinogenic properties of the extracts of medicinal plants have been demonstrated in many studies, there are studies showing that all these properties can be reversed with the increase of the applied dose and that plant extracts can be genotoxic or even carcinogenic depending on the dose [16]. In a study conducted with extracts of two different plant species (P. aviculare and Plantago L.) belonging to the genus Plantago, it was reported that plant extracts did not decrease lymphocyte cell division ability, but only at a high concentration of 2000 µg/ml, it decreased the cell division ability by more than 50% [7]. In our study, it is thought that plant extract exerts its healing effects in DNA-damaged cells by activating DNA repair mechanisms or by activating enzymes and antioxidants that scavenge increased free radicals in damaged cells.
The Genoprotective Effects of Plantago lanceolata Extract and Luteolin-7-B-D-Glycoside on Lymphocyte and HEPG-2 Cells

The anticarcinogenic effects of medicinal plants are widely studied. In our study, Plantago L. extract exerted a cytotoxic effect against cancer cells (HEPG-2) and had an anticarcinogenic effect. The genotoxic effect of high-dose plant extract application was found to be higher than the low dose. In HEPG-2 cells with DNA damage with H2O2, all doses of Plantago L. reduced the DNA damaged cells by showing an antigenotoxic effect. Different Plantago species are used by the public for cancer treatment. P. major ssp. the leaves of the plant are boiled and used in cancer patients [17]. Studies have reported that Plantago L. extracts significantly suppress cell proliferation in various cancer cells, depending on the dose [5,18,19]. Similar findings have been shown in an in vivo animal study [20].

In our study, it was investigated by comet analysis whether the dose-dependent antigenotoxic effect of the plant extract is through LUT-7G flavonoid, which is one of the main components of the plant. Accordingly, in healthy human lymphocyte cells, in line with the plant extract findings, only the comet parameters due to DNA damage increased in the high-dose LUT-7G group. In lymphocytes with DNA damage with H2O2, all doses of LUT-7G showed a healing effect and revealed its antigenotoxic potential. In some studies similar to our study, it has been demonstrated with many genotoxicity tests, including comet, that LUT-7G has a genoprotective effect on DNA damaged cells. In these studies, it has been suggested that the healing effects of LUT-7G stem from its radical scavenging activities [21]. In our study, only high dose of LUT-7G showed genotoxic effect. The maximum dose of LUT-7G we applied is much higher than the doses in the studies in question. There are many studies in the literature showing that flavanoids may be mutagenic and genotoxic at high doses [22]. It is known that high-dose flavonoids can induce DNA damage by binding to metal ions in cells, depending on the pro-oxidant activity and by inhibiting the catalytic activity of topoisomerase II [23].

In our study, LUT-7G exerted an anticarcinogenic effect by showing cytotoxic effects against HEPG-2 cancer cells at all doses applied. There are many studies on the anticarcinogenic properties of LUT-7G. LUT-7G reduces the proliferation and migration of cancer cells and induces apoptosis [24]. Luteolin usually stops the development of cancer by stopping cell division at the G1/S or G2/S checkpoint [25]. In cancer cells, the PI3K/Akt signaling pathway plays an important role in the apoptosis of tumor cells. In a study, it was shown that Akt phosphorylation decreased and the expression of apoptotic genes increased as a result of application of 10 and 30 μM LUT-7G to GC cells. In addition, it has been reported that LUT-7G can cause the disintegration of the cytoskeleton by accelerating the degradation of F-actin, thus causing the mesenchymal phenotype, and also by inhibiting the Notch 1 signal pathway, reversing the Epithelial-Mesenchymal Transformation (EMT), thus reducing the invasion and migration ability of the cells [8]. With desmutagenic effect of some flavonoids against carcinogens in various cancer cells, that is, after DNA damage; It has been reported to show an antimutagenic effect by neutralizing mutagen or with radical scavenging activity after DNA damage [26]. Although the antimutagenic mechanism of action of LUT-7G is not fully understood, it is suggested that it affects various metabolic pathways such as glycolytic enzymes or protein synthesis and the function of topoisomerase I and II 23,27].

5. CONSEQUENTLY

When the literature data and the results obtained from this study are brought together, the methanol extract of the Plantago L. and the LUT-7G flavonoid are not genotoxic; on the contrary, they are anticarcinogenic in cancer cells, possibly through antioxidant mechanisms or by blocking the interaction of important macromolecules in cell transformation mechanisms. The validity of the results obtained from the study is limited by the cell types used and the application doses of the test substances. It is recommended that Plantago L. and LUT-7G be studied at different doses and in different cell types to obtain more information on their genotoxicity/antigenotoxicity and carcinogenicity/anticarcinogenicity potential.

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Ethical approval

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