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Cytotoxic Effects of Probiotic Bacteria *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* Grown in the Presence of Oleuropein on Human Prostate Cancer Cells

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Abstract

Functional foods are groups of foods that have potentially positive effects on health beyond basic nutrition. Probiotics are beneficial microorganisms, being as a group of functional foods, and when ingested in adequate amounts, they possess positive health effects to host organisms including having anti-carcinogenic functions, yet their exact mechanisms how they possess anti-carcinogenic activities are still under investigation. Another group of functional foods is plant-based phenolic compounds. They also have potential to possess anti-carcinogenic activities. Among them, oleuropein is very interesting and well-known phenolic compound naturally present in olive fruits and olive oil. As the phenolic compounds and probiotic bacteria can be present at the same time in human gastrointestinal tract, this study aims to investigate the *in vitro* cytotoxic effects of *Lactobacillus acidophilus* LA-5 (LA-5) and *Lactobacillus rhamnosus* GG (GG) grown in the presence of oleuropein on human prostate cancer cell line. For this, oleuropein was added to media of these bacteria and effects of cell-free supernatants of these combinations (phenolic+probiotic) on cytotoxicity of prostate cancer cell line PC-3 were investigated using MTT Method. Different concentrations of oleuropein were added to bacterial cultures and oleuropein added to the growth medium of LA-5 had additional effects on the cytotoxicity of cell-free supernatants of this bacterium. However, addition of oleuropein to the growth medium of GG did not show significant changes on the cytotoxicity of cell-free supernatants of this bacterium.
Thus, the results indicate that new combinations of functional foods have potential to formulate new nutraceuticals.

Keywords: Cytotoxicity, MTT Assay, Phenolic compounds, Probiotics, Prostate Cancer, Oleuropein.

1. Introduction

Functional foods are groups of foods having potentially positive effects on health beyond basic nutrition [1]. Probiotics, a group of functional foods, are microorganisms that have positive effects on human health when administered in sufficient amounts [2].

Lactic acid bacteria are the group of probiotic microorganisms investigated in detail for health benefits [3]. The best known probiotics are *Lactobacillus acidophilus* and *Lactobacillus rhamnosus*. One of the best studied beneficial bacteria is *Lactobacillus rhamnosus*, which is available as a dietary supplement and added to a variety of foods, such as dairy products. It belongs to the genus *Lactobacillus*, a type of bacteria that produces the lactase enzyme. This enzyme converts sugar lactose found in dairy products into lactic acid. *L. rhamnosus* can survive in acidic and alkaline conditions in the human body and has many potentials and uses for the digestive system and other health areas. *L. acidophilus* produces antimicrobial agents such as hydrogen peroxide (H_2O_2), diacetyl and bacteriocin as well as organic acids. These substances inhibit degrading organisms and foodborne pathogens. It has been found that *L. acidophilus* inhibits various bacterial strains by producing bacteriocin [4]. Probiotic bacteria are expected to adhere to the mucus layer, form colonies

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and multiply. Adherence to the cell wall in the intestine is an important feature for colonization in the digestion [5]. *L. acidophilus* can be found in more parts of the intestine compared to other bacteria [6].

Another group of functional foods is plant-based phenolic compounds. Humans have been using extracts of olive leafs for medicinal purposes for centuries [7]. As a traditional 'Mediterranean Diet', olive and olive oil are the most famous and popular diet components [7]. Olive leaf extracts, olive fruits, and olive oil contain tocopherols, carotenoids, phospholipids, and phenolic compounds. Oleuropein is considered as main ingredient in olive tree and in all other constituent parts of the fruit (peel, pulp, and seed) [8]. It is one of the secoiridoids, a specific group of coumarin-like compounds [7]. It is a glycosylated compound, produced in secondary metabolisms of the plants (Figure 1) [9]. It reaches more than 140 mg/g in dried immature olives and reaches 60-90 mg per g of dried leaves [9]. It is a very intensively studied phytochemical for its health benefits and for its medicinal usage [7]. Although most of the studies related with oleuropein and its health effects have been performed *in vitro*, some human and animal trials used 'Mediterranean diet' to show its effects on the health. Anti-oxidant tests showed that oleuropein potentially inhibits oxidation of low-density lipoproteins induced by copper sulfate [10], to scavenge nitric oxide [11] and the free radical 1,1-diphenyl-2-picrylhy-drazyl (DPPH) [12]. Furthermore, oleuropein has a strong anti-microbial activities against different microorganisms, including Gram(+) and Gram(-) bacteria, and mycoplasma [13–15]. Using different cancer cell lines, oleuropein showed anti-carcinogenic activities. Oleuropein and its aglycone inhibited *in vitro* growth of glioblastoma, erythroleukemia, renal cell adenocarcinoma, malignant melanoma of the skin-lymph node metastasis,ductal carcinoma of the breast pleural effusion, colorectal adenocarcinoma, and breast cancer [16, 17].

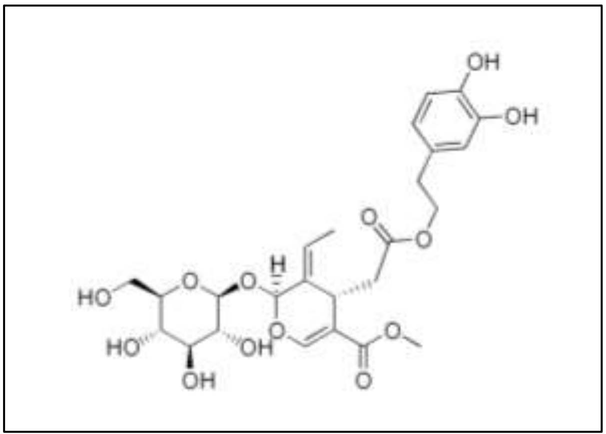

Figure 1. Chemical structure of oleuropein.

This study aimed to reveal the *in vitro* cytotoxic effects of probiotic-oleuropein combination on PC-3, a prostate cancer cell line. Thus, different concentrations of oleuropein were added to bacterial cultures and it was observed whether these concentrations showed any inhibition on *L. acidophilus* LA-5 (LA-5) or *L. rhamnosus* GG (GG). Then, cell-free supernatants of the probiotic bacteria grown on the presence of oleuropein were used to investigate its cytotoxic activity on PC-3 cell line.

2. Materials and Methods

2.1. Growth of probiotic bacteria in the presence of Oleuropein

Lactobacillus acidophilus LA-5 and *Lactobacillus rhamnosus* GG, which are kind gifts of Chr. Hansen, Turkey, were grown in Man, Rogosa and Sharpe (MRS) medium without shaking, at 37ºC [18]. The bacteria were divided into groups and treated with oleuropein. Oleuropein was not added to the control group (MRS only), and 50-250 µg/mL oleuropein was added to the treated groups in MRS medium. Bacteria were sub-cultured three times before the experiments.

2.2. Cell Culture

In the present study, androgen receptor negative human prostate cancer cell line PC-3 was used to investigate the effects of probiotics grown in the presence of oleuropein. Cells were grown in RPMI-1640 medium (containing %10 FBS, 0.1 mg/mL streptomycin, and 100 U/mL penicillin) in 75 cm^2 culture flasks in a humidified CO_2 incubator (%5 CO_2 + %95 O_2 at 37°C). 3-(4,5-dimetiltiazol–2-il)-diphenyl tetrazolium bromide assay (MTT Assay) was used to investigate the effects of cell-free supernatants of probiotics grown in the presence of oleuropein on prostate cancer cells [19].

2.3. MTT Method for Cytotoxic Investigation

PC-3 cells were seeded into 96-well plates ($15x10^3$ cells/well). The cells were treated with 10 times and 50 times diluted cell-free supernatants of probiotics grown with oleuropein (250 µg/mL) for 24 h at 37°C. 0.5 mg/mL MTT working solutions in sterile PBS, were added to each well, followed by incubation for 24 h (37°C). Then, excessive MTT was removed, washed with PBS, solubilized with DMSO, and OD at 550 nm was read with ELISA reader. Cell-free supernatants of probiotics grown without oleuropein were taken as control, while the absorbance of the cells treated with MRS medium without any growth diluted with RPMI-1640 was taken 100% cell viability [20]. The cell viabilities were determined by absorbance obtained from treated wells proportioned to the control absorbance values.

2.4. Statistical Analysis

Each experiment was conducted as at least three biological replicates and three technical replicates. All data were represented as mean ± S.D. Student's *t*-test was used to evaluate the significance in differences between treated and control groups. The significant differences were accepted when $p<0.05$.

3. Results and Discussion

Three different concentrations (50, 100, and 250 µg/mL) of oleuropein were used in the growth cultures of probiotic bacteria *L. acidophilus* LA-5 and *L. rhamnosus* GG and their effects on growth, surface hydrophobicity, and auto-aggregation properties of the bacteria were evaluated previously [21]. None of the oleuropein concentrations inhibited the growth of the bacteria. This indicates that even though oleuropein has significant inhibitory effects on pathogenic bacteria, it does not affect the probiotic growth, thus it has a potential to selectively inhibit the bacteria, making a good candidate for new formulations of functional foods, as well as to further study the interactions between oleuropein and probiotics [13, 14].

We previously showed that the surface hydrophobicity of LA-5 was significantly reduced ($p <0.05$) at 50 µg/mL concentration of oleuropein and increased at 100 µg/mL concentration [21]. In contrast, at only 100 µg/mL, it ($p <0.05$) decreased the surface hydrophobicity of GG bacteria compared to the control group. Bacterial surface hydrophobicity (*i.e.* adhesion to hydrophobic solvents) is an important feature for bacteria to retain in the gastrointestinal tract [22]. This is one of the factors providing better adhesion of probiotic bacteria to the mucosa. In the present study, it was found that oleuropein reduced or increased the surface hydrophobicity of LA-5 depending on the dose. Increased cell surface hydrophobicity at a concentration of 100 µg/mL may enable these bacteria to colonize the intestinal tract better to demonstrate their probiotic activity. Furthermore, although it is undesirable that the surface hydrophobicity of GG bacteria is reduced at the same concentration, other factors are important for the attachment of bacteria to the mucosa [23].

Auto-aggregation results showed there is statistically significant ($p <0.05$) decrease in aggregation of LA-5 bacteria at only 50 µg/mL concentration of oleuropein [21]. Bacterial aggregation is defined as the clustering of bacteria by adhering to each other [24]. The high amount of auto-aggregation may lead to better adhesion [25]. However, other factors that play a role in adhesion to the mucosa are proteins in the surface layer of bacteria, particularly S-layer proteins [26]. In this respect, the effects of oleuropein on mucosal adhesion of probiotic bacteria and also on surface proteins should be examined.

Prostate cancer is a leading health problem within the male individuals and has a high death rate [27]–[29]. Table 1 shows cell-free supernatants of probiotics possess *in vitro* cytotoxic activities against human prostate cell line PC-3.

When LA-5 grown without oleuropein, its cell free supernatant had no significant cytotoxic effects; however, when oleuropein added to the growth medium, the supernatant significantly ($p<0.05$) lowered the cell viability (Table 1). On the other hand, we wanted to show whether oleuropein itself has a cytotoxic effect, the results indicated that oleuropein does not possess any significant alteration on the cell viability. This indicates that LA-5 may metabolize oleuropein so that the metabolite can further lowered the cell viability. It is known that when oleuropein metabolized, hydroxytyrosol can be produced and hydroxytyrosol has anti-tumorigenic activities [30]. The metabolite of oleuropein, hydroxytyrosol, has been found to lower the viability PC-3 cells [31]. Once PC-3 cells were treated with hydroxytyrosol (80 µmol/L), it enhanced superoxide level and apoptotic cell death was induced [31]. It also showed a dose-dependent proliferation inhibition of prostate cancer cells (LNCaP and C4-2), by inducing G1/S cell cycle arrest, inhibiting cyclins cdk2/4 and D1/E, as well as inducing p21/p27 [32].
Cell-free supernatants of GG showed very high inhibition of PC-3 cell viability ($p<0.05$). There was no additional effect when oleuropein added to the growth medium.

Cytotoxicity results indicate that beneficial interactions between oleuropein and *L. acidophilus* LA-5 have been observed, thus when oleuropein is ingested through the diet, it is possible to be metabolized by *L. acidophilus* into more bioactive compounds. Or, new formulations including oleuropein and *L. acidophilus* can be more beneficial when compared to either bacteria or phenolic compound alone.

Natural compounds are of paramount importance as alternative drug investigations are gaining attention. Studies have showed that either plant extracts or pure compounds could be potential candidates for the development of novel therapeutic anticancer compounds [16, 17, 33-35]. Furthermore, lactic acid bacteria are known to be effective against cancer cells *in vitro* [20]. However, combination of two potential therapeutic agents (i.e. phenolics and probiotics) could be more effective as bacteria can modulate the bio-active compounds, as well bio-active compounds can modulate the probiotics, which has been shown in the present study.

Table 1. Cytotoxic effects of cell-free supernatants of probiotics grown with oleuropein (250 µg/mL) on prostate cancer PC-3 cells. The values are the mean of three biological replicates, given with standard deviation in parenthesis.

***L. acidophilus* LA-5**	**Relative Viability Percentage (S.D.)**
1/10 Diluted Medium Control	100 (9.06)
1/10 Diluted Control (without oleuropein)	87.32 (34.27)
1/10 Diluted Supernatant	57.14 (29.41) [a, b]
1/50 Diluted Medium Control	100 (8.99)
1/50 Diluted Control (without oleuropein)	93.80 (6.86)
1/50 Diluted Supernatant	86.66 (7.40) [a, b]
***L. rhamnosus* GG**	**Relative Viability Percentage (S.D.)**
1/10 Diluted Medium Control	100 (9.06)
1/10 Diluted Control (without oleuropein)	5.16 (0.92) [a]
1/10 Diluted Supernatant	5.54 (1.60) [a]
1/50 Diluted Medium Control	100 (8.99)
1/50 Diluted Control (without oleuropein)	83.96 (10.57) [a]
1/50 Diluted Supernatant	75.11 (18.65) [a]
Oleuropein (250 µg/mL)	**Relative Viability Percentage (S.D.)**
1/10 Diluted	96.88 (4.82)
1/50 Diluted	95.57 (3.11)

[a] Statistically significant ($p<0.05$) when compared to Medium Control.
[b] Statistically significant ($p<0.05$) when compared to Control (without oleuropein).

4. Conclusion

The present study aimed to gain insight into *in vitro* cytotoxic activities of combination of oleuropein and probiotic bacteria against prostate cancer cells. Oleuropein has dose-dependent effects on probiotic properties like auto-aggregation and surface hydrophobicity, as well the combination may show additional inhibitory activities against prostate cancer cells.

In conclusion, oleuropein and probiotic bacteria have the potential to increase the benefits of each other through a synbiotic interaction.

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Author's Contributions

Hasan Ufuk Celebioglu: Designed the study, drafted and wrote the manuscript, performed the experiment and result analysis.

Busenur Celebi: Performed the experiment, result interpretation and helped in manuscript preparation.

Yavuz Erden: Performed the experiment and result analysis, and helped in manuscript preparation.

Emre Evin: Performed the experiment, result interpretation and helped in manuscript preparation.

Orhan Adali: Assisted in analytical analysis on the structure, supervised the experiment's progress, result interpretation and helped in manuscript preparation.

Ethics
There are no ethical issues after the publication of this manuscript.

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