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The European beech leaves extract has an antibacterial effect by inducing oxidative stress

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Abstract

Folk medicine has been widely spread for centuries and, in recent years, was proven to be very effective against a great variety of diseases. In this context, the aim of this study is to explore the European beech (Fagus sylvatica L.) leaves as a new alternative source of polyphenolic compounds. The leaves used in this study were harvested in September from the Sinaia region. The leaves were dried, then grinded and polyphenols were extracted using ethanol 70% (v/v). Afterwards, polyphenols and flavonoids were assayed and identified accurately by HPTLC. Antimicrobial activity of the extract was tested against Escherichia coli, one of the most common microorganisms that cause urinary tract infections. The test for antimicrobial activity consisted of the agar diffusion method, growth curves and assessment of the activities of some important enzymes for the bacteria cells such as superoxide dismutase, catalase and glucose 6-phosphate dehydrogenase. The Fagus sylvatica L. leaves were proven to be rich in polyphenols and to have a moderate antimicrobial activity against Escherichia coli ATCC 8739, most probably due to the oxidation of these compounds which increases the oxidative stress in the culture media leading to irreversible damage to the bacterial cells.

Keywords: *Fagus sylvatica* L., antibacterial activity, polyphenols

1. Introduction

Since ancient times people have used plants to treat themselves for any diseases from the common cold to systemic illnesses. However, medicinal plants have started to be intensely studied in the last decades, proving their numerous benefits and multiple biological effects. Nowadays, it is established that secondary plant metabolites, such as polyphenols, flavonoids, vitamins, tannins, etc., are good for human health and they are an important source for new natural medicines (S. SHRESTHA & al. [41]). Polyphenols are produced by plants in order to protect themselves from pathogens and UV radiation (K.B. PANDEY & al. [32]) and they were proven to have anti-inflammatory, antibacterial (S. THUSOO & al. [43]), antioxidant and antiviral (J.M. CALDERON-MONTANO & al. [5]) properties, but they can also be used for reducing triglycerides levels or in cardiovascular diseases (W.T. CHANG & al. [6]). More often, polyphenols and flavonoids are used to treat bacterial infections, local or systemic, among the polyphenols with the most powerful effect being quercetin (S.G. DASTIDAR & al. [10]), caffeic acid (H.F. HARRISON & al. [17]) or chlorogenic acid (H.C. KARACA & al. [23]). Another important aspect of using polyphenols for treatment is that these compounds

are relatively easy to procure, they are cheap and they rarely cause allergenic reactions, immunosuppression or hyper-sensitive reactions (B.K. DASH & al. [9]).

Fagus sylvatica (European beech) is a common tree for temperate and warm regions of the Northern Hemisphere that can be found only in Europe, Asia and North America because it is not resistant to drought, aridity or frost (F. CLINOVSCHI & al. [7]). The *Fagus sylvatica* L. leaves were shown to contain catechins, cis-coniferin and cis-syringin that have antifungal properties (P.V. PETRAKIS & al. [34]), but also manganese, molybdenum, copper, zinc, iron, cobalt ions and sulphur compounds (B. MANKOVSKA & al. [30]), saponins, ginsenoside derivatives (G. ROMUSSI & al. [39]) and vitamins C, E, K or á-tocopherols (K.J. KUNERT & al. [25]). There are references to the use of European beech leaves for the treatment of fever, diarrhoea, skin liver or respiratory diseases (CRACIUN [8]) or to the antibacterial effect of these leaves against *Helicobacter pylori* (M. FREDERICH & al. [15]).

Escherichia coli is a Gram-negative bacterium with an aerobic metabolism, saprophytic and conditionally pathogen. Oxygen is vital for the cells, but it can also become toxic if the concentration of reactive oxygen species (ROS) such as O_2^- or H_2O_2 increases and oxidative stress sets in. In order to prevent such an event, the bacterial cells synthesize specific enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase that inactive the harmful ROS (H. DOI & al. [12]). *E. coli* is normally found in the human intestinal flora; however it can cause serious infections in circumstances such as mutations or translation to a different environment, leading to intestinal infections, urinary tract infections or, in more severe cases, endocarditis, meningitis or septicemia (L.M. DITU & al. [11]).

Antibiotics are the most common treatment for such cases, but unfortunately drug-resistant bacteria are now spreading more and more and epidemics are even more dangerous when they reach less developed countries or immunocompromised patients (I.M.P. VIOLANTE & al. [44]). Urinary tract infections are almost exclusively caused by this bacterium and are very common nowadays. This is why scientists are constantly searching for new remedies, especially given the high rate of drug-resistant strains (S.S. JUSTICE & al. [22]).

The aim of the present study was to evaluate the antibacterial effect of a polyphenlic extract from *Fagus sylvatica* L. leaves against *Escherichia coli* ATCC 8739, a Gram-negative bacterium that is the most frequent cause of urinary tract infections and to compare this effect with one used antibacterial agent, methylene blue (MB).

2. Materials and Methods

Plant material

Fagus sylvatica L. *folium* raw material was harvested from Romanian Carpathian Mountains, Sinaia region. The taxonomic identification was done by the botanists team of National Institute of Chemical-Pharmaceutical R&D (INCDCF), Bucharest, Romania. A voucher specimen (FSAM20-24) has been deposited in INCDCF *Plant Material Storing Room*. Studies were carried out on beech leaves harvested in September time; the leaves were shade dried and minced as medium-size plant powder.

Obtaining and characterisation of the *Fagus sylvatica* L. *folium* extract

The extract was obtained as follows: 100 g powder of beech leaves (L. PIRVU & al. [35]) were twice (heat-assisted, 1 hour, continue stirring) extracted with 1000 mL of 70% (v/v) ethanol prepared with deionized water. The resulting pairs of extracts were filtered through paper filter resulting in 1700 mL beech leaves ethanolic extract further used for analytical studies.

For microbiological studies, in order to avoid false positive results, the ethanolic extract was concentrated at residue, which was solved into 20% propylene glycol reagent in a manner to assure 5 mg total phenols (gallic acid equivalents) per 1 mL sample.

Total phenols content was estimated using Folin-Ciocalteu assay. Briefly, three aliquots of 25 to 50 ěL test sample were treated with 200 ěL of Folin-Ciocalteu reagent then finished at 5000 ěL with 5% (w/v) sodium carbonate. Flasks were mixed and left at room temperature for 5 minutes then the absorbance was read at 750 nm. Total phenols content was estimated by using gallic acid (ref.) standard calibration curve (r^2=0.9997) and the results expressed as total phenols, mg (GAE) / 1 mL test sample (M. GONZALEZ & al. [16]).

Total flavones content was estimated using the following method: three aliquots of 50 to 100 ěL test sample were treated with 600 ěL of 2.5% aluminium chloride and 1000 ěL of 10% sodium acetate (w/w) then finished at 5000 ěL with (50%, v/v) ethanol. Mixtures were incubated for 30 minutes at room temperature then the absorbance at 410 nm was read. Rutin flavonol has been used as standard compound and calibration curve subject matter (r^2=0.988) and the results expressed as total flavones, mg (R) / 1 mL test sample (A.J.D. FERNANDES & al. [14]).

The HPTLC method was used for the identification of the compounds found in the extract. Briefly, volumes measuring from 0.5 to 3 ěL test vegetal product (FSAM20-24) as well as reference samples (mixtures of 3-5 *Fluka* and *Sigma-Aldrich* phenolics) were loaded as 8 mm band length in the 10 × 10 cm silica gel 60F HPTLC plate (Merck, Darmstadt, Germany) using Linomat 5 CAMAG instrument (Muttentz, Switzerland). The loaded plate was then kept in TLC twin developing chamber at 18–19°C with the mobile phase (ethyl acetate–acetic acid–formic acid–water/100:12:12:26) up to 90 mm. The developed plate was dried using a hair dryer and then immersed into identification reagents (Natural Product followed by PEG4000). The dried plate was next disposed in photo-documentation chamber, and the images were captured at UV 366 nm. Spots' assignment has been done by using reference compounds data and plant product literature data as well (H. WAGNER & al.[45]; E. REICH & al. [37]).

Inoculum preparation

The *E. coli* ATCC 8739 was grown on casein soya agar medium (Merck, Germany) (CaSoA). The strain was activated by culturing the cells on CaSoA and incubated for 18-24 hours at 30-35°C. Growth curves were obtained using casein soya broth (Merck, Germany).

Growth curves

Afterwards, the liquid medium was inoculated with 10^4-10^5 CFU (colony forming units)/mL. The optical density of the medium was read using a densitometer (Biosan, Letonia) at 600 nm at 60 minutes intervals, both for a control sample and for samples in which different concentration of polyphenols were added as it follows: 25 µg GAE/ 1 mL sample, 50 µg GAE/ 1 mL sample, 100 µg GAE/ 1 mL sample respectively. Also, as a reference, in different test tubes, was added methylene blue (Merck, Germany) to the bacterial culture in two concentrations: 25 µg/mL and 50 µg/mL in order to assess the activity of the beech leaves extract against this compound.

Antimicrobial assay using the diffusion method

The tests were performed in sterile Petri dishes, each containing 15-20 ml of culture medium previously inoculated with 10^4-10^5 CFU/ml. On each dish, 4 stainless steel cylinders of 8 mm diameter were placed on the solidified surface of the medium. Afterwards, in each cylinder were added 0.2 ml in the following manner: one cylinder contained 20% propylene glycol (the solvent in which the plant extracts were prepared) and the other 3 contained the test sample (plant extract). The Petri dishes were incubated 18-24 hours at 30-35 °C. After the incubation period, the growth inhibition zones were measured and the results were expressed as mean of three independent measurements.

Enzymatic assays

In order to assay some intracellular enzymes, 3 Erlenmeyer flask containing 200 mL liquid medium that was inoculated with 10^4-10^5 CFU/mL were used as it follows: the first one was the control sample, in the second one polyphenolic extract was added to a final concentration of 50 µg/mL and in the third one methylene blue was added to a final concentration of 50 µg/mL. The optical density was read at 600 nm until the concentration of bacterial cells reached a value of approximate 10^9 CFU/mL (OD=1.0395) and the culture medium was centrifuged at 5000 rpm for 10 minutes, the pellet was resuspended in 0.9% NaCl and washed twice. In order to break the bacterial cell walls a Mini bead-beater apparatus (Retsch MM301, Retsch GmbH, Germany) was used to agitate the cells at a 30 Hz frequency for one minute, 10 cycles were performed. The obtained suspension was centrifuged at 5000 rpm for 10 minutes to remove the cellular debris. Afterwards, the whole extract was carefully transferred in clean tubes and used for further assays. The protein assay was carried out using the Lowry method (O.H. LOWRY & al. [27]) and afterwards individual enzyme activities were determined.

Glucose 6-phosphate dehydrogenase (G6PD) activity was measured according to X.H. Yuan & al. [46]. Briefly, in a quartz spectrophotometer cuvette were mixed 670 ěL HEPES buffer 50 mM, 20 ěL glucose-6-phosphate 40 mM, 10 ěL $NADP^+$ 30 mM and 50 ěL protein extract. The rate of the NADPH formation was a measure of G6PDH activity, and this was be followed by means of the increase in extinction at 340 nm. Catalase activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm (R.F. BEERS & al. [4]). 600 ěL phosphate buffer 0.1 M were mixed with 350 ěL H_2O_2 0.059 M and 50 ěL protein extract.

Catalase specific activity was calculated in terms of U/mg protein, where 1U is the amount of enzyme that catalyzed the conversion of 1 ěmol H_2O_2 in a minute.

Superoxide dismutase (SOD) was assayed according to the method based on NADPH oxidation (F. PAOLETTI & al. [33]). 160 ěL TBD buffer pH 7.4 were mixed with 5 ěL EDTA-$MnCl_2$ 100 mM/50 mM, 20 ěL protein extract, 8 ěL NADH 7.5 mM and 20 ěL â-mercaptoethanol 100 mM, atferwards the decrease in absorbance at 340 nm was followed for 10 min to allow NADPH oxidation by the generated superoxide anion. One unit of SOD activity was defined as half-maximal inhibition.

Statistical analysis

All experiments were carried out in triplicates and the results are expressed as mean values ± standard deviation (SD) of three measurements.

3. Results and Discussions

Several other studies regarding plants extracts with antimicrobial activity showed that various extraction systems may be used, such as methanol, hexane, water, chloroform or ethanol. The polyphenolic extract used in this study had a concentration of 33.55 mg/g GAE (gallic acid equivalents) polyphenols and 5.72 mg/g RE (rutin equivalents) flavonoids. As individual compounds, using the HPTLC method we described earlier, were identified numerous chlorogenic acid and apigenin derivatives added to catechins, kaempferol and quercetin derivatives (L. PIRVU & al. [36]).

To determine the antibacterial effects of the extract we applied different microbiological and biochemical techniques and we selected *Escherichia coli* strain because it is a Gram-negative bacterium usually found in the intestinal human flora, but it can also become pathogen, leading to urinary tract infections or even infections of the intestinal mucosa.

The diffusion method for determination of antimicrobial activity refers to the measurement of the growth inhibition zone diameter that appears as a result of the action of the natural compounds from the polyphenolic extract. However, this method can only be used

carefully and keep in mind that there is always the possibility that an extract may contain large molecules that can migrate with difficulty in the agar (M. AFSHARZADEH & al. [1]).

Our results, a inhibition zone diameter of 12 mm (L. PIRVU & al. [36]), are comparable with the results from a previous study that tested ethanolic extracts from *Thymus vulgaris* (thyme) and *Piper nigrum* (black pepper) against *E. coli* O157:H7 and reported inhibition zone diameters of 13 mm and 15 mm (M. ZARRINGHALAM & al. [47]). Another study revealed that the extracts in methanol and hexane from *Rhus succedanea* (Japan wax) lead to diameters of 15 and 18 mm against *E. coli* MTCC 723 (S. SHRESTHA & al. [41]). The 12 mm diameter zone, however, was a better result than those shown by methanolic extracts from *Juniperus excelsa* (juniper sp.), *Juniperus oblonga* (juniper sp.), *Platycladus orientalis* (oriental thuja) against *E. coli* PTCC 1330, of 6 mm, 6 mm and 7 mm (M. AFSHARZADEH & al. [1]) or the aqueous extract from *Moringa oleifera* (Moringa tree) which gave an inhibition zone of only 7 mm against *E. coli* DSM 8579 (T. MARRUFO & al. [37]).

Once we established the antimicrobial activity of this extract against *E. coli* we tested smaller doses of polyphenols, *Fagus sylvatica* L. *folium* extract dilution series respectively, with the purpose to determine the mechanism through which they act. There were tested three dilution series meaning total phenols content of 25, 50, 100 µg GAE/mL sample respectively, and also two concentrations of MB chemical reference, 25 and 50 µg/mL sample, respectively.

As it can be observed in Figure 1, in the presence of test extracts, beech leaves dilution series, the bacterial cells develop slower, the doubling time increases, the lag period decreases, the culture reaches rapidly a plateau phase, all of which lead to the conclusion that the European beech leaves extract has an antimicrobial effect against *Escherichia coli* ATCC 8739. There should be mentioned that the extract is not colourless, it contains different compounds that can also absorb light, that being the reason why the OD values are higher in the samples than in the control medium. Although it is unanimously accepted that specific plant polyphenols have certain antimicrobial effects, the mechanism through which they act is still disputed. Some studies showed that some of them, such as caffeic acid or gallic acid, have an increase affinity for the plasmatic membrane of bacterial cells, they can penetrate the lipidic bilayer and disturb its structure, phenomena that can lead to the death of the cells (T. HASHIMOTO & al. [18]).

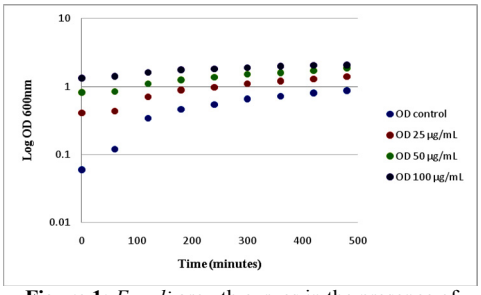

Figure 1: *E. coli* growth curves in the presence of *Fagus sylvatica* L. leaves extract

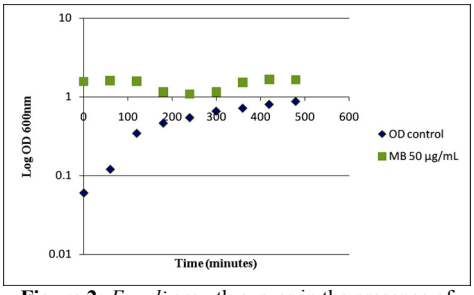

Figure 2: *E. coli* growth curves in the presence of methylene blue

In comparison, we assayed the activity of a known chemical antimicrobial agent - methylene blue, a thiazine dye and the results are shown in Figure 2. In this case, there can be observed a slightly decrease of the optical density values in the middle of the time period studied, probably due to the fact that methylene blue is transformed in corresponding leucoderivatives by the bacterial dehydrogenases, reaction that can be easily visualised because the colour of the culture media switches from blue to green (ROMEO-TEODOR [38]).

Methylene blue is a lipophilic dye that can penetrate the plasmatic membrane of bacterial cells and can then be reduced by NAD(P)H dehydrogenases in the presence of molecular oxygen, thus generating ROS that can lead to oxidative stress (H. ATAMNA & al. [3]). On the other hand, it is well known that polyphenols are chemical compounds with antioxidant activity, therefore are likely to interact with the same membrane reductases as MB. In particular, the dihydroxyphenolic groups from polyphenols may form complexes with metallic ions, especially catechins and their derivatives, that are attracted by the negative charged lipopolysaccharides from the structure of Gram-negative bacterium and in the presence of oxygen, these ions can accept an electron, process that ends with H_2O_2 formation, an oxidative stress inducer, stress that can lead to the death of the bacterial cells (N. HOSHINO & al. [20]). This is why we suggested the possibility that the polyphenols from the European beech leaves ethanolic extract may exert their antimicrobial activity through oxidative stress induction. To test this hypothesis, we obtained a total proteic extract from culture cells non-treated, treated with the new extract and treated with methylene blue and assessed the activity of some enzymes involved in oxidative stress management, such as superoxide dismutase, catalase and glucozo-6-phosphatase dehydrogenase. We chose to assess the antibacterial activity of the European beech leaves extract against the activity of MB, a well known antibacterial agent used in the treatment of septic shock induced by the presence of bacterial lipopolysaccharides or pulmonary infections caused by bacterial endotoxins (E.S. KWOK & al. [26]).

The assay of protein concentration revealed a decrease both in the presence of polyphenolic extract and methylene blue. We observed that there is an almost 50% difference between the concentration of control and the two samples, from 9.77 mg/mL to 6.95 mg/mL in the presence of European beech leaves extract (with 50 ěg GAE/mL sample) and 7.5 mg/mL in the presence of MB. Catalase is the enzyme responsible for the removal of peroxide hydrogen from the bacterial cells, that appears from the molecular oxygen when the cells are submitted to oxidative stress (K. MAETA & al. [29]), which seems to be the case here. We can see that the specific activity of catalase presented a slightly increase (Figure 3). There can be observed that the enzymatic activity increases with approximately 20% in the case of the beech leaves extract treatment, and it increases a lot more in the presence of methylene blue, with as much as 60%. A similar study (M. AKAGAWA & al. [2]) showed that peroxide hydrogen, a substrate for catalase, is involved in the process of oxidative deamination of polyphenols, which is responsible for the release of a high concentration of ROS, this being probably the reason why the catalase activity only increases moderately. As we said before, the extract from *Fagus sylvatica* L. leaves contains a series of catechinic derivatives that have the capacity to reduce O_2 and lead to formation of H_2O_2, which induces oxidative stress on fungus and bacterial cells (K. MAETA & al. [32]).

Also, our previous studies (L. PIRVU & al. [35]) on beech leaves ethanolic extract and corresponding aqueous, ethyl acetate and chloroform fractions isolated through processing the whole extract, an augmented pro-oxidant activity of the non-polar compounds found in chloroform fraction was revealed *versus* high antioxidant potency of the polar compounds, found in aqueous and ethyl acetate fractions respectively, explaining beech leaves ethanolic extract anti-oxidant and pro-oxidant capacity as well. Moreover, our studies (L. PIRVU & al. [36]) clearly demonstrated this cooperation between polar and non-polar compounds by revealing the lack of activity of separate fractions (aqueous, ethyl acetate and chloroform respectively) versus certain activity (12 mm diameter of inhibition zone) of the origin, beech leaves ethanolic extract.

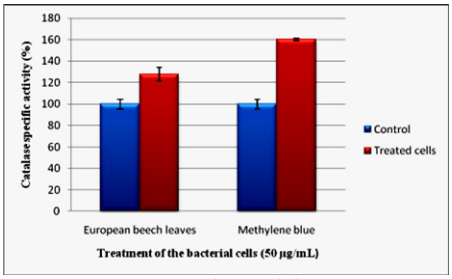

Figure 3: Catalase activity assay

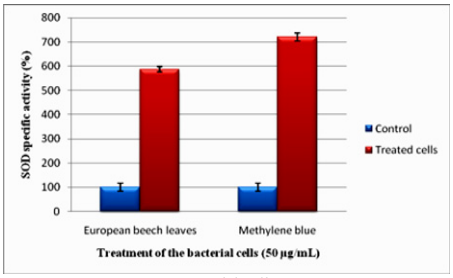

Figure 4: Superoxide dismutase assay

By comparison, methylene blue determined a higher increase in the enzymatic activity of catalase, probably because its capacity to go back and forth between oxidised and reduced state, a cycle that is maintained by the molecular oxygen from the media and that generates ROS (H. ATAMNA & al. [3]). Regarding the activity of superoxide dismutase (SOD), its values increased considerably in both cases, as it can be seen in Figure 4. As it can be observed, the specific activity of SOD increased dramatically with approximately 500%, 600% respectively, comparative with the control. These differences that can be explained by the exacerbation of some mechanism that lead to the formation of superoxide radicals, the substrate for SOD.

Polyphenols can penetrate the lipidic bilayer where they can suffer auto-oxidation processes in the presence of molecular oxygen, and that can lead to the destabilisation of the plasmatic membranes. Even more, they can form complexes with metallic ions such as copper or iron by reducing them: Cu(II) to Cu(I), Fe(III) to Fe(II), reactions that determine the formation of superoxide anions (A.H. SMITH & al. [42]). Such differences regarding the specific activity of SOD lead to the conclusion that the bacterial cells are subjected to oxidative stress conditions due to the formation of ROS that lead to the death of the cells by DNA damage or disturbing protein integrity. In order to protect itself, *E. coli* possess a series of reglons, such as *soxRS* (superoxide radical response) that determines the increase in the synthesis of enzymes such as superoxide dismutase, endonuclease IV or glucose 6-phosphate dehydrogenase (K.A. HOPKIN & al. [19]).

It is obvious that there is not a major contrast between the activation of SOD by the polyphenolic extract or MB, and it can be explained by the fact that both compounds interact with the plasmatic membrane, where they suffer oxidation processes that generate superoxide anions, a toxic compound for the bacterial cells, so they try to counter balance by increasing the synthesis of SOD (S.Y. KIM & al. [24]).

Glucose 6-phosphate dehydrogenase (G6PD) plays an essential role not only in the pentose phosphate way, but also in the generation of reduced equivalents in the form of NADPH that have an important role in the defense against ROS by maintaining the intracellular redox state and repairing the lesions caused by ROS (B.E. LUNDBERG & al. [28]). We assayed the activity of glucose 6-phosphate dehydrogenase, a key enzyme in glucose metabolism to determine if this was affected in any way by polyphenols or methylene blue.

As it can be seen in Figure 4, the activity of this enzyme increases significantly only in the presence of methylene blue, and almost indiscernibly in the case of the extract from European beech leaves. A possible explanation for this is that MB, once inside the cell, is oxidised and it leads to the transformation of NADPH to NAD^+ and $NADP^+$, the last one being a co-factor for G6PD, and its higher concentration leads to the activation of the enzyme (J.M. MAY & al. [31]). Methylene blue induces the use of pentose phosphate way for the metabolism of glucose, so an increase in the activity of G6PD was to be expected. This enzyme is regulated

by the *zwf*, whose activity is regulated by three mechanisms: the regulation of the growth rate (D.L. ROWLEY & al. [40]), the action of *MarA* (multiple antibiotic responses) reglon (K.W. JAIR & al. [21]) and by the action of *soxRS* reglon, which determines the induction of G6PD synthesis in oxidative stress conditions (W.P. FAWCETT & al. [13]).

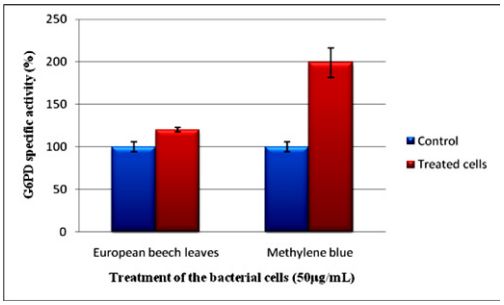

Figure 5: Glucose 6-phosphate dehydrogenase (from *E. coli*) activity assay in the presence of *Fagus sylvatica* L. leaves extract and methylene blue

4. Conclusions

The present study focused on the antibacterial activity of an polyphenolic extract from European beech leaves against *Escherichia coli* ATCC 8739. We found that the bacterial cells develop slower in the presence of polyphenols, which proves there is an interference with their metabolism. Also, the assay of protein concentration supports the claim that the extract, at small concentration, slowes down the cell development. We suggested that polyphenols may react with some membrane reductases, interference that leads to cell death and our assays revealed that some enzymes as catalase, SOD or G6DP have a higher activation rate after the cells have been exposed to the polyphenols, which can be explained by their increased potential to generate ROS.

Summarizing all of these results, it was clearly revealed the certain antimicrobial activity of the compounds found in European beech leaves raw material against *Escherichia coli* pathogenic bacterium and the mechanism based on simultaneously, pro-oxidant and antioxidant potency of the polar and non-polar compounds found in European beech leaves whole extracts. These results may be useful for obtaining new hygiene or cleaning natural products.

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