Biological analysis of *Ginkgo biloba* extract

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Abstract. *Ginkgo biloba* belongs to the Ginkgoaceae family and contains a large variety of compounds. The genetic diversity, tree age and soil type influences the yield of chemical compounds found in different parts of the plant. This study performs phytochemical and antibacterial analysis of ethanolic extracts from *Ginkgo biloba.* The leaves have been used in order to obtain *Ginkgo biloba* tincture. They were dryed in the shadow. The quality index was 1:5, the concentration of ethanol being 70 %. Based on this tincture a qualitative and quantitative phytochemical analysis was performed through thin layer chromatography and high performance liquid chromatography. By thin-layer chromatography the qualitative flavonoids, such as quercetin and rutin from *Ginkgo biloba* extract, have been identified. By high performance liquid chromatography with reversed phase the quercetin quantity from *Ginkgo biloba* leaves was assessed and that is 1.34 mg/mL. The plant extracts were tested on Gram negative bacteria *Escherichia coli* and on Gram positive bacteria *Staphylococcus aureus*. The extracts presented an inhibition about 17.5 mm on *S. aureus* and about 10.5 mm on *E. coli*. The ethanolic extracts exhibited a stronger activity versus *S. aureus* strain, which demonstrates that the quercetin extracts can be used in treatment of different infections caused by these bacteria.
Key words: quercetin, *Ginkgo biloba*, thin-layer chromatography, high performance liquid chromatography with reversed phase, bacterial susceptibility.

Rezumat. *Ginkgo biloba* aparţine familiei Ginkgoaceae şi conţine o varietate mare de compuşi. Diversitatea genetică, vârsta arborelui și tipul de sol influențează randamentul compușilor chimici în diferitele părţi ale plantei. Acest studiu constă în analiza fitochimică şi antibacteriană a extractului vegetal de *Ginkgo biloba.* Pentru a obţine tinctura etanolică s-au folosit frunzele de *Ginkgo biloba*. Acestea au fost uscate la întuneric. Indicele de calitate a fost de 1:5 iar concentrația de etanol a fost de 70 %. Această tinctură obţinută a fost supusă analizelor fitochimice calitative şi cantitative prin cromatografie în strat subţire şi cromatografie lichidă de înaltă performanţă cu fază inversă. Prin cromatografia în strat subţire au fost identificate calitativ flavonoide, precum quercetina şi rutina, din extractul de *Ginkgo biloba*. Prin cromatografia lichidă de înaltă performanţă cu fază inversă s-a stabilit cantitatea de quercetină din extractul de frunze de *Ginkgo biloba,* aceasta fiind de 1.34 mg/mL. Extractul vegetal a fost testat pe bacterii Gram negative *Escherichia coli* şi pe bacterii Gram pozitive *Staphylococcus aureus*. Extractul a prezentat o inhibiţie în medie de 17.5 mm pentru *S. aureus* şi în medie de 10.5 mm pentru *E. coli*. Extractul etanolic a prezentat o activitate mai puternică la tulpina *S. aureus*, ceea ce demonstrează că extractul cu quercetină poate fi utilizat în tratatea diferitelor infecţii cauzate de aceste bacterii.
Cuvinte cheie: quercetină, *Ginkgo biloba*, cromatografie în strat subţire, cromatografie lichidă de înaltă performanţă cu fază inversă, susceptibilitate bacteriană.

Introduction. *Ginkgo biloba* is a dioecious tree with a rich canopy. It belongs to the Ginkgoaceae family. The leaves have a gray, yellow-greenish or yellow-brown color. The leaves are collected by hand from trees, and for industial cultures the leaves are collected with equipment used for cotton harvesting. The yellow color of the leaves indicates a high quantity of secondary metabolites. The quality of the leaves depends on the time of harvesting. It plays an important role in the yield of terpenoids. It was found that the percentage of bilobalide and ginkgolides from leaves was lowest in spring and

reached a maximum yield at the end of summer or in the beginning of autumn; however their concentration dropped until the leaves have fallen. Some factors, for example genetic diversity, tree age, and soil type influence the yield of chemical compounds found in leaves (van Beek 2003). It is among the oldest tree species on Earth. It is native to China. This tree is grown for ornamental purposes and due to its therapeutic importance. *Ginkgo* tolerates temperate climates including zones that are temperate cold and temperate Mediterranean (Denis et al 2012).

Ginkgo biloba contains a variety of compounds. The presence of terpenes has been demonstrated in different parts of the plant. It has also been observed that light can influence the accumulation of terpene compounds. Hue & Staba (1993) have shown that the biosynthesis of gingkolides held in leaves and roots. Other possible factors which may affect the ginkgolides and bilobalide diversity include the soil type, developmental stage, age, etc. They observed that the leaves of trees older than 30 years contain smaller amounts of ginkgolides and bilobalide than seedlings of three years; and contents of terpenes was much higher in leaves, if at every harvest the trees were cleaned (Sohier & Courtois 2008).

Extracts of leaves in solvents such as acetone and ethanol contain 22-27% flavonoids (quercetin, kaempferol, isorhamnetin), 5-7% terpenes (of which 2.8-3.4% ginkgolides A, B and C and also 2.6-3.2% bilobalides, and less than 5 mg/kg ginkgolic acids (Chan et al 2007).

Ginkgolides are diterpenes, which together with bilobalides are considered to be the primary active components. Ginkgolides A, B, C and M have been isolated from the root shell and ginkgolides A, B, C and J from leaves. The only difference among these compounds is the number and position of hydroxyl group (OH) which may be present at C1, C3 or C7 of the spiropentane framework (van Beek 2003).

Bilobalides are sesquiterpene compounds. It was observed that the concentration of bilobalide and ginkgolides was very low in the spring season and highest in midsummer. In late autumn when the leaves become yellow the bilobalide concentration begins to decrease (van Beek 2003; Tang & Eisenbrand 2013). The bilobalide have insecticidal properties and protect the tree against the phytophagous insects and mammals (Chan et al 2007).

In the HPLC analysis of the extracts from *Ginkgo biloba* leaves was observed that the primary peaks are represented by flavonoids like quercetin and kaempferol (Fig. 1) and minor peaks are represented by aglycone, apigenin and luteolin (van Beek 2003).

The flavonoids are found in the leaf epidermis, stored in vacuoles (Hu et al 2010). In plants, flavonoids play an important role as pigments by protecting plant against UV radiation, this explains that the fluorescent light and also UV radiation increases the enzymatic activity and production of quercetin and kaempferol. The flavonoids also protect the plant from infections (Bücherl 2013).

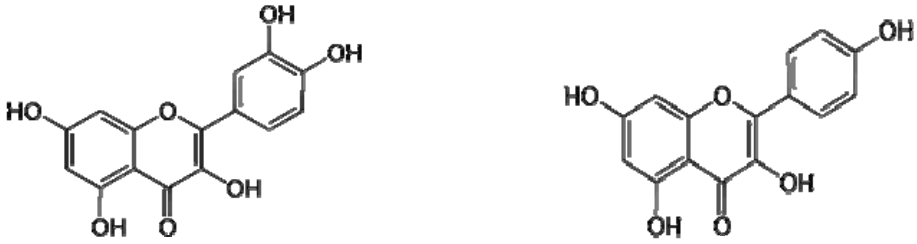

Figure 1. Structure of main flavonoids present in the *Ginkgo biloba* extract: left (kaempferol), right (quercetin).

Different extracts of leaves of *Ginkgo* shown a increased inhibition on *Bacillus subtilis* strains, *Xanthomonas phaseoli*, *Agrobacterium tumefaciens* and *Escherichia coli*. Also, it has been observed that the methanol extract showed more inhibition, followed by ethanol extract, chloroform, and the hexanoic one (Sati 2011). In Romania a study has been made on 144 chicken carcasses, which presented infection with *Camphylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* at a rate of 15-23 %. On these microbial

strains a resistance to tetracycline, sulfonamides and quinolones was observed (Dan 2015). The diseases resulting from eating contaminated food with pathogenic bacteria are a priority care preoccupation for public health (Kordali 2005). *Ginkgo* essential oil showed an inhibitory and bactericidal effect against pathogenic strains: *Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes, Escherichia coli and Salmonella typhymurium.* A higher inhibition was observed for Gram positive bacteria. The results obtained by Bajpai (2015) have shown that the essential oil obtained from *Ginkgo biloba* leaves is capable to disrupt the functions of the membrane of Gram positive and negative bacteria causing significant morphological changes in the wall of pathogen agents. Damage of the membrane is followed by increased adenosine 5' triphosphate on the extracellular space at cells treated with essential oil. There has also been a leakage of potassium ions when they were exposed to *Ginkgo* essential oil. Efflux of potassium ions was higher at Gram positive bacteria (Bajpai 2015). Leakage of intracellular material is a phenomenon induced of many antimicrobials substances which causes cell death (Farag 1989). The antimicrobial effect can be due to inhibition of proton motive force, inhibition of mitochondrial respiration, electron transfer chain, inhibition of substrate oxidation, loss of metabolites, disruption of DNA, RNA synthesis, proteins, lipids, and polysaccharides (Denyer & Hugo 1991).

Materials and Methods

1.Extraction protocol from Ginkgo biloba. The leaves have been used in order to obtain *Ginkgo biloba* tincture, and they were dryed in the shadow. The quality index was 1:5, the concentration of ethanol being 70%. The leaves from *Ginkgo biloba* along with the alcohol mixture were stirred, cleaned and left to macerate for five days in the concentration of ethanol established. During this time the density was determined. It was then filtered and after the analysis it was sealed. To analyze compounds from extract of *Ginkgo biloba* the German Pharmacopoeia 2012 was used (HAB 2012). *Ginkgo biloba* extract was obtained from PlantExtract in Rădaia, Cluj County.

2.Quercetin identification from Ginkgo biloba extract by TLC. Thin layer chromatography (TLC) is a qualitative method which consists in dividing into three equal areas on the chromatographic plate and resemble of the compounds in the mixture with specific standards or by identifying compounds based on the values of retention factor. Thin layer chromatography can also be a quantitative method by correlating the concentration with the peak area. In order to analyze the compounds of the *Ginkgo biloba* extract by TLC a silica gel plate was used with a flourescent indicator of 254 nm, with a thickness of 0.25 mm and 7x20 cm dimensions. The migration distance is 150 mm, and the eluent is a mixture of ethyl acetate, acetic acid, formic acid and water (67.5: 7.5:7.5:17,5 v/v). From the extract, 20 µL were applied. As standards were used quercetin (1 mg/mL), rutin (1 mg/mL) dissolved in 10 mL of methanol and than applied in volumes of 10 µL. The plate was dried at 100-105 ºC and was sprayed with 1% aminoethanol difenilborat in methanol. After 30 minutes the chromatogram was viewed under flourescence at 365 nm.

3.Identification of quercetin out of Ginkgo biloba extract using HPLC with reversed phase. High Performance Liquid Chromatography (HPLC) with reversed phase was used according to the protocol presented in HAB (2012) (in the present study stationary phase is non polar and the mobile phase is polar). From crushed leaves samples 2.5 g were mixed with 50 mL of acetone 60% vol. for 30 minutes. It was filtered off and then the vegetal material was extracted with another 40 mL acetone 60% vol. The filtrates were mixed and filled up to 100 mL with 60% acetone vol. From the total volume, 50 mL was evaporated to eliminate acetone and then it was brought quantitatively into a 50 mL flask using 30 mL methanol. 4.4 mL of 10% hydrochloric acid was added and 50 mL with purified water was completed. The mixture was centrifuged. Then 10 mL of the supernatant was placed in a brown round bottom flask of 10 mL, sealed, and heated for 25 minutes in a water bath. Then it was cooled to room temperature. In the HPLC programme with the reversed phase the time and quantity injected were set. The HPLC column was silicagel C18, 120 x 4 mm, 5 µm and the mobil

phase was represented by A-phosphoric acid pH=2; B-methanol. The injection volume was 10 µL and the debit was 1 mL/minute. As a standard, quercetin (0.5 mg/mL) was used and the detection was 370 nm.

4.Disk paper method for testing the bacteria sensibility to Ginkgo biloba extract; qualitative diffusimetric method. The susceptibility of the tested bacteria (*Staphylococcus aureus* ATCC 25923, Gram positive and *Escherichia coli* ATCC 25922, Gram negative) to the plant extract was determined using a paper disc diffusion assay on Nutrient Agar plates (Atlas 2010), following the method described by Carpa et al (2014). Bacterial suspensions were adjusted to 0.5 McFarland turbidity (1-2 x 10^6 cfu mL^{-1}) and spread evenly over the entire surface of the agar plates using a sterile cotton swab. The plates were allowed to air-dry for approximately 10 minutes before the paper disc (6 mm) was placed on the agar plate. Each extract test was replicated two times. The plates were incubated at 37 ºC for 24 hours. For each microorganism tested, zones of inhibition of growth were examined, and the diameter of each zone was recorded.

Results and Discussion

1.Thin-layer chromatography (TLC) analysis of Ginkgo biloba extract. By thin-layer cromatography we identified qualitatively the compounds from the *Ginkgo* extract. The plate for TLC is split into three zones (lower part, middle part and the upper part) in which specific colored bands appear for the tested flavonoid and for other active compound in *Ginkgo* extract.

Ginkgo biloba extract was applied in 20 µL from the sample. The eluent consisted of an ethyl acetate mixture, anhydrous formic acid and purified water. We used quercetin and rutin as standards, from which 10 µL were applied. After the migration of the compounds the plate was sprayed with aminoethanol diphenylborate (in methanol) and poliethilenglicol (in ethanol). After 30 minutes the chromatogram was observed in flouroscent light at 365 nm.

Standard chromatogram shows in the lower part a brown-yellowish band for rutin and in the upper part a yellow band for quercetin. Sample chromatogram shows in the lower part a yellow band for rutin, in the middle part a blue and yellow band and in the upper part there are several pale bands: yellow, green and a intense yellow band for quercetin (Fig. 2).

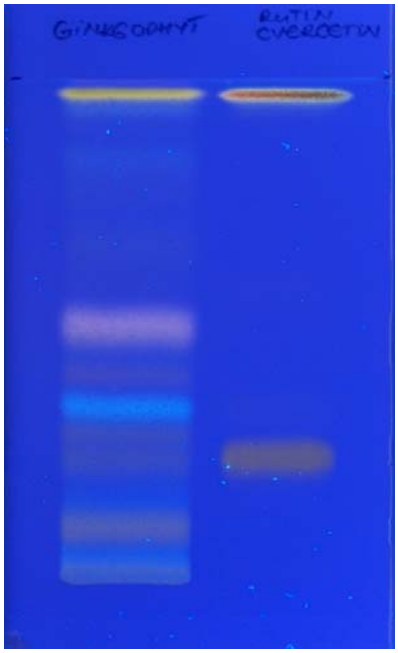

Figure 2. The chromatogram plate with *Ginkgo biloba* extract.

Based on a number of bands and their intensities in Fig. 2 it can be concluded that the extract contains a large number of compounds, which are found in different quantities. Therefore, the extract contains a large quantity of flavonoids expressed in quercetin. Also there is an appreciable quantity of biflavonoide, namely rutin.

2.Analysis of Ginkgo biloba extract by HPLC with reversed phase. For ethanolic extract of *Ginkgo biloba* leaves, the main flavonoid is quercetin and the areas that were obtained are shown in Table 1.

Table 1

The quantity and the areas of quercetin

Quercetin quantity (mg/mL)	*Quercetin areas*
0.01	250441
0.02	594638
0.03	1506259
0.04	2595416
0.1	4832624
0.15	7988679

Based on the areas, a calibration curve is formed for quercetin and this represents the peak based on concentration (Fig. 3).

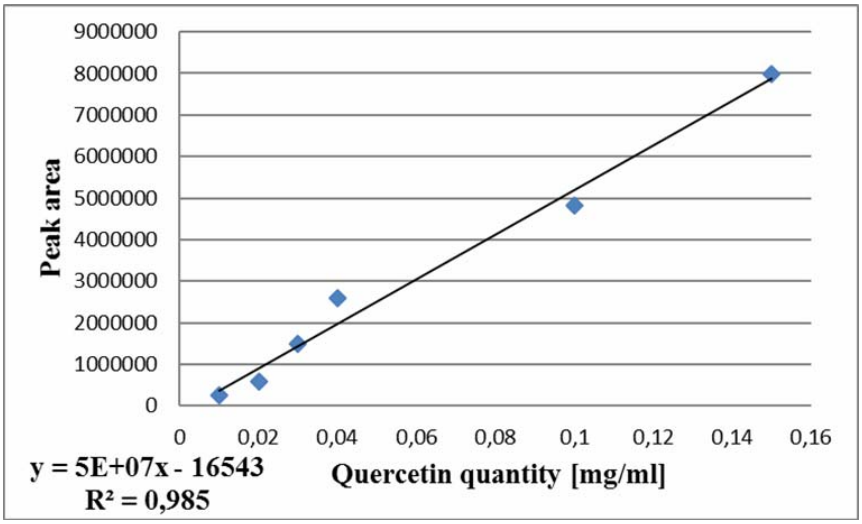

Figure 3. Calibration curve for quercetine from leaf of *Ginkgo biloba* at 370 nm.

The linear equation was determined, where x represented the concentration [mg/mL], and y is the peak area of the sample. Afterwards the quantity of the compounds from *Ginkgo biloba* ethanolic extract was calculated to find out peak area from sample. Peak area for quercetin was taken from extract of *Ginkgo* chromatogram. In Figure 4 the chromatogram for quercetin standard, at 370 nm.

From the chromatogram of quercetin standard a spectrum of its absorption at 370 nm was recorded (Fig. 5).

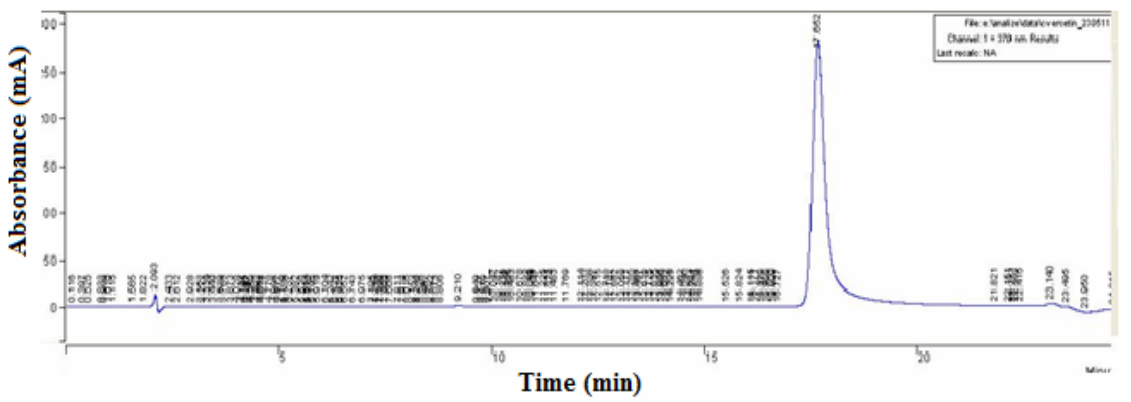

Figure 4. Quercetin standard curve in reversed phase HPLC at 370 nm.

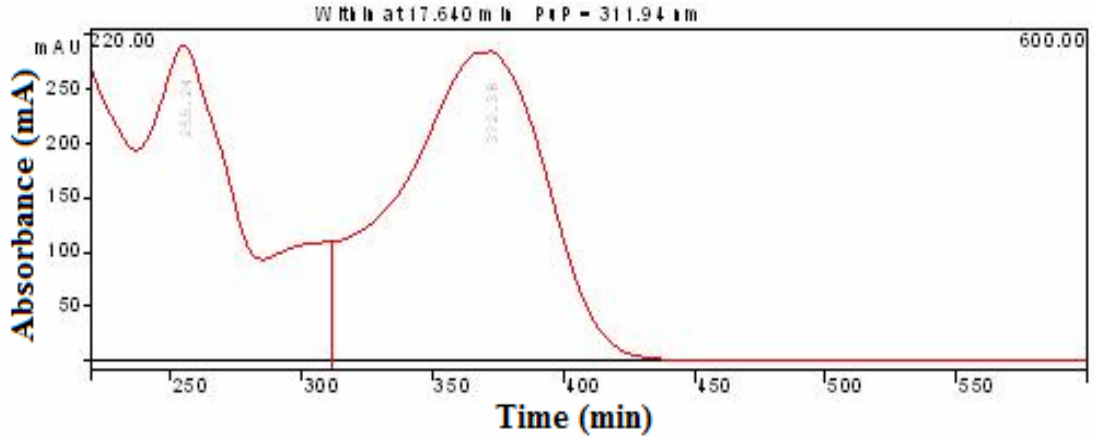

Figure 5. Quercetin standard spectrum at 370 nm.

Based on standard chromatogram and its spectrum it was estimated that the quercetin from the sample will come out around the minute 17-18. After the injection of the *G. biloba* leaf extract its chromatogram was obtained. In Fig.6 the overlapped chromatograms of standard quercetin and ethanolic extract of *G.biloba* leaves are shown.

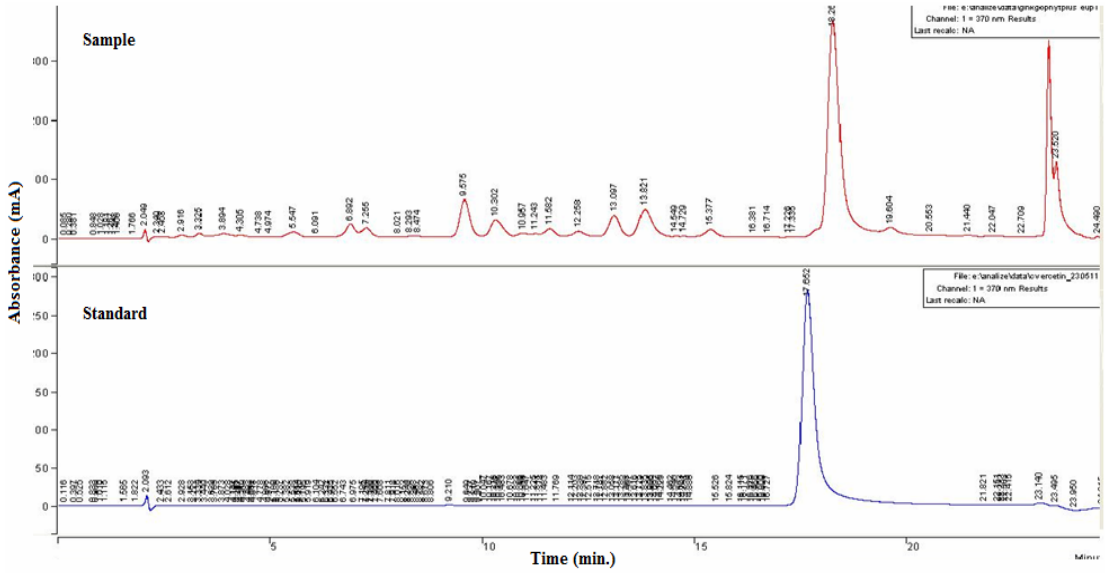

Figure 6. Overlapped chromatograms of quercetin from standard and leaves of *G.biloba.*

In Figure 7 the absorption spectrum of quercetin in ethanolic extract from the leaves of *G.biloba* is presented.

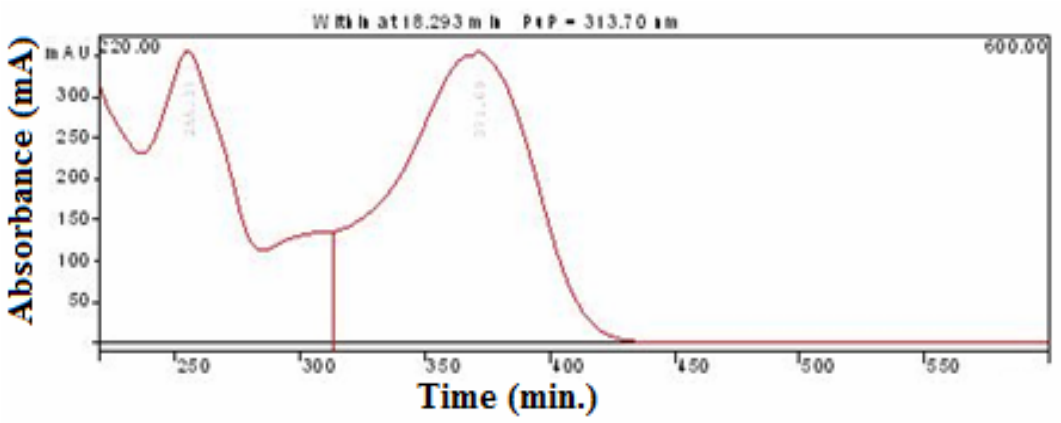

Figure 7. Spectrum of ethanolic extract of leaves from *G. biloba* (quercetin).

In the ethanolic extract from *G. biloba* leaves a peak of absorption at 18.289 minutes for quercetin was observed. These results compared with the quercetin absorption spectra showed that the substance is correctly identified. By the equation obtained from the curve of the calibration standards and depending on peak area the flavonoid quantity (expressed as quercetin present in 1 mL of the sample) was also calculated.

The quercetin area from the sample of 18.289 minutes read at 370 nm is 80023888. X= 1.34 mg/mL quercetin.

3.Testing the bacteria susceptibility at G. biloba extract. On the Nutrient Agar culture media (Atlas 2010) in aseptic conditions the test strains were inoculated (*Staphylococcus aureus* - Gram positive and *Escherichia coli* - Gram negative). At the inoculation of the test microorganism 1 mL of bacterial suspension was used, which was spread on the whole surface of the media and the culture surplus was eliminated. After the inoculated cultures dried off, the 6 mm disks were applied (Fig. 8).

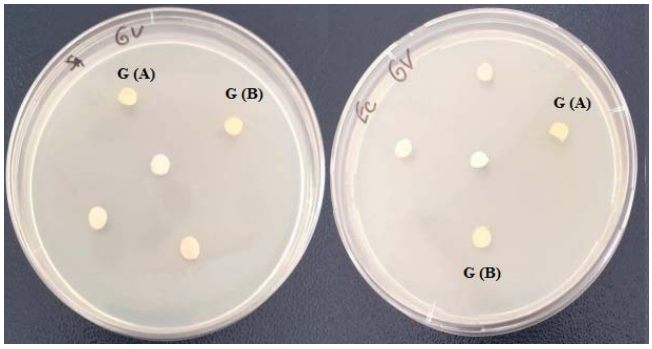

Figure 8. Applying disk papers on NA culture medium inoculated with *S. aureus*, *E. coli* (Middle = control (ethanol 70%); G (A, B) = *G. biloba* extract).

The culture media was incubated 18-24 hours, and than the inhibition zone that appeared was measured (Fig. 9). At the Gram positive strain (*S. aureus*) it was observed that the sensibility is very high on all the tested probes with the biggest inhibition diameter of 18 mm.

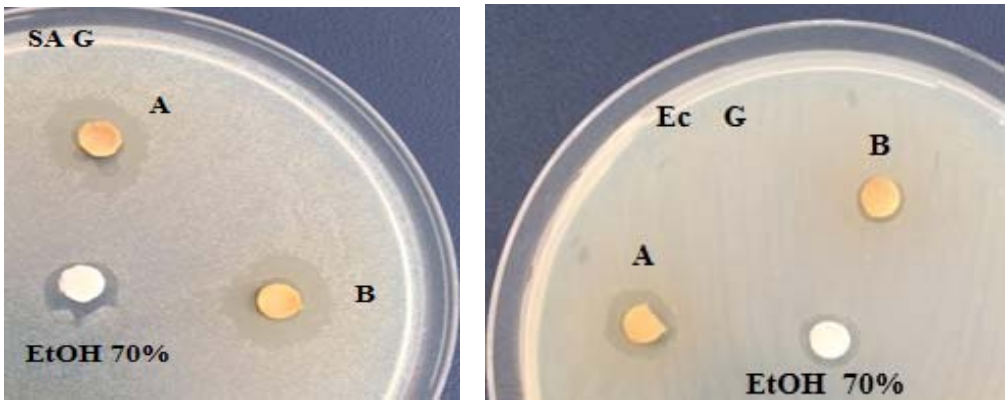

Figure 9. Bacterial sensibility of *S.aureus* (SA G) and *E.coli* (Ec G) at the disk papers with *G. biloba* after 24 incubation hours (EtOH = control (ethanol 70%); A, B = *G. biloba*).

On the ethanolic extract of *G. biloba* the inhibition zones were determined after 24 hours both at the Gram positive strain and at the *E. coli* Gram negative strain. On these, the sensibility was slightly lower than on *S. aureus*, reaching a diameter about 10.5 mm (Fig. 9). The diameter of the inhibition zones of the *G. biloba* plant extract measured on the inoculated plates with the test strains *S. aureus* and *E. coli* is presented in Table 2.

Table 2

Inhibition zone diameter (mm) of disc soaked with extract

Sample tested	*Inhibition zone diameter (mm)*	
	Staphylococcus aureus ATCC 25923	*Escherichia coli* ATCC 25922
Blank (EtOH 70%)	9.5 mm	9 mm
Ginkgo biloba	17.5 mm	10.5 mm

Conclusions. *Ginkgo biloba* is a dioecious tree which belongs to the Ginkgoaceae family and contains important flavonoids such as quercetin, that has an antioxidant action. This is found in the epidermis of the leaf and is stored in the vacuole. *Ginkgo* ethanolic extract was obtained from dried leaves with a concentration of 70% ethanol.

By thin-layer chromatography (TLC) the flavonoids such as quercetin and rutin from extract of *G. biloba* were qualitatively determined.

By high performance liquid chromatography with reversed phase (HPLC) the quercetin quantity from *G. biloba* leaves was identified and that is 1.34 mg/mL.

In order to test the sensitivity of *Escherichia coli* and *Staphylococcus aureus* bacteria the 6 mm disk paper method soaked with 40 µL extract was used. After the incubation it was observed that the extract of *Ginkgo* showed a very high strength in all tested samples, and it obtained an average diameter of 17.5 mm. In the case of *E. coli* strain a slightly decreased resistance was observed at *Ginkgo* extract and an average diameter of 10.5 mm was obtained. Not only identified compounds (quercetin) are responsible for the antimicrobial activity but also all secondary metabolites present in the extract.

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