Correlation of the Theoretical Study with the Experimental Determination of the Antibacterial Effect of *Vaccinium myrtillus folium* (VM-f) Plant Extract

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The last century was marked by efforts to search for natural compounds with antibacterial therapeutic properties, due to the gradual reduction of the number of effective allopathic antibiotics and on the toxic effects of antibiotic residues in animal products. Medicinal plants are considered new resources for producing agents that could act as alternatives to antibiotics in the treatment of antibiotic-resistant bacteria. We determined the polyphenolic composition of the tested plant extract by HPLC technique. The aim of this study was to evaluate the antibacterial activity of Vaccinum myrtillus folium (VM-f) ethanolic extract, using the diffusion method on nutritive agar (Kirby-Bauer). We compared the experimental results with the theoretical antibacterial activity calculated with AUTODOCK 4.2.

Keywords: antibacterial effect, Vaccinium myrtillus folium, molecular docking

Today bacterial infections are a major cause of death rate, both among humans and animals. The phenomenon of antibiotic resistance is frequently encountered lately, an increasing number of bacterial species being able to develop mechanisms of resistance to the action of classical antimicrobial agents. The multidrug resistance phenomenon grows, the bacteria being resistant to all commonly used chemotherapies [1].

Currently, more than half of the bacteria that cause nosocomial infections are resistant to at least one of the classical antibiotics. In the case of an infectious process, a first-line antibiotic is administered, with bioavailability, efficacy and high tolerance. If the infectious agent is resistant to it, a second-line antibiotic with a broader spectrum is administered [2]. Nosocomial infections with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* are resistant to the second and even third line of antibiotics, being classified as multi-drug resistant germs [3].

Some bacteria are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs. The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years, scientists explore plants for phytochemicals that can be developed for the treatment of infectious diseases [4].

Nowadays, according to the WHO, natural therapies are widely used by the world's population [5], because medicinal plant parts are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins with remarkable pharmacological effects [6]. *In vitro* studies have shown that plants have antibacterial efficacy against many bacterial species, revealing the importance of natural resources, little studied in this respect, in the fight against bacterial resistance and the destruction of bacterial agents [7].

In vitro studies performed by Gibbons (2004) and Braga *et al.*, (2005) proved that many plant extracts may be efficient on *Staphylococcus aureus* strains, revealing the importance of this unexploited resource in antibiotic resistance of pathogen agents [8]. Polyphenols are important secondary metabolites constituents of plants, being demonstrated that a regular consumption of them help us to fight infections caused by various pathogens [9].

Bilberry contains a variety of phenolic compounds, including flavonols (quercetin, catechins), tannins, ellagitannins, and phenolic acids, and anthocyanins [10].

The plant extract from VM-f has antibacterial potential on *Escherichia coli* and *Proteus vulgaris*, being frequently used in the treatment of urinary tract infections. It also has antibacterial effect on *Staphylococcus aureus* and *Salmonella enterica* [11].

Pure phenolic compounds were found to inhibit only gram-negative bacteria (*Salmonella* species and *Escherichia coli*), and the effects were related to the degree of hydroxylation of the pure phenolic compounds. Berry extracts were found to inhibit those affected by the pure phenolic compounds; further, extracts inhibited the growth of not only *H. pylori* and few gram positive microorganisms: *Bacillus*, *Clostridium*, and *Staphylococcus*. In conclusion, whole berries or extracts of whole berries may be more effective as antimicrobials than purified extracts, due to the synergism of component potentiation [12].

The present study aimed at investigating the antimicrobial potential of the VM-f plant extract, taking as a reference the recognized antibacterial effect of the choice antibiotics (control +) on the reference strains of: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603). As a negative control, we used ethyl alcohol, used in the preparation of plant extracts.

In the theoretical part we modeled and optimized ligands with Gaussian software suite. We used computational docking technique to predict bound conformations and free energies of binding for antibiotics and apigenol molecule ligands to macromolecular targets, using AUTODOCK 4.2.

Experimental part

Material and method

Plant material

The leaves of *Vaccinium myrtillus* were harvested from the Botanical Garden of Craiova City (Dolj County, Romania) and were air-dried in the shade at room temperature.

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Preparation of plant extract

The plant product was used in the form of tincture, obtained by simple percolation, in a plant / solvent ratio (ethanol 70o) of 1: 5 (F.R.X.). The test sample of each tested tincture is found in the Pharmacognosy Laboratory Collection of the Faculty of Pharmacy in Craiova [13].

Analysis by HPLC chromatography technique of flavonoids and polyphenol carboxylic acids of tincture

HPLC analysis was performed in specific working conditions: HPLC Jasco MD-2015, two-pump, thermostat, UV-DAD detection system, degassing system; eluent A (acetonitrile); eluent B (0.1% phosphoric acid); working gradient: prerun →10% A, 90% B; 13.1 min. →22% A, 78% B; 14.1 min. → 40% A, 60% B; 20.1 min. → 40% A, 60% B; 50 mPA pressure; detection: 330 nm; retention times [min.] for flavonosids, flavonoid aglycones and polyphenol carboxylic acids: chlorogenic acid - 7.12, caffeic acid - 7.964, ferulic acid - 13.147, rutoside - 15.19, isoquercitrin - 15.68, rosmarinic acid - 17.58, apigenin-7-glucoside - 17.65, quercetol - 18.71, kaempferol - 20.25 [14].

Determination of antibacterial effect

The test microorganisms are derived from standard reference strains purchased from the Cantacuzzino Institute, classified as sensitive to the action of the antibiotics of choice [15].

Into petri dishes pour 25 mL of nutrient agar (Mueller-Hinton), 100 mm diameter, making a uniform layer of 3-4 mm. The culture medium should have a *p*H of 7.2 and a composition suitable for the proper development of the bacterial species to be tested. The test germs come from standard reference strains. Preparation of the inoculum was performed by suspending 2-3 standard colonies in physiological saline, the turbidity being controlled nephelometrically. Sowing was carried out by introducing a buffer into the bacterial suspension, followed by squeezing to remove excess inoculum over the entire surface and uniformly erasing an average of three times. Drying the inoculated plates is achieved by holding for 10 minutes at room temperature (22 ° C) before the samples are deposited. Pre-sterilized filter paper sterilized sheets (ø = 6 mm) were impregnated with a volume of 25 μL of the test plant extract and then kept at the oven for 24 hours to evaporate the ethyl alcohol. The blank discs are prepared under the same conditions. Sample deposition was performed immediately after drying, about 15 minutes after sowing, using an ophthalmic pens, applying each sample to the culture medium surface (disk diffusion method). The standard antibiotic (control +) discs, the alcohol-wetted discs (the blank) and the disc imbedded with the test plant extract are deposited 1.5 cm from the edge of the petri dish and 3 cm apart from each other .

The antibiotic-impregnated (control +) discs were chosen based on the bacterial species sensitivity, being produced by BD (USA).

- amoxicillin - *Staphylococcus aureus* (ATCC 25923), gram-positive cocci 20μg;
- levofloxacin - *Escherichia coli* (ATCC 25922), bacillus gram (-), 5 μg;
- amikacin - *Proteus vulgaris* (ATCC 6380), bacillus gram (-), 30 μg;
- ceftazidime - *Pseudomonas aeruginosa* (ATCC 27853), bacillus gram (-), 30 μg;
- cefotaxime - *Klebsiella pneumoniae* (ATCC 700603), bacillus gram (-), 30 μg;

Incubation was carried out for 18 h at 37°C in the upturned position of the Petri plate. The reading of the results was performed by eye using a graduated ruler, measuring the diameter of the inhibition zone (mm), induced by the test samples. Concurrent bacterial development occurs on the surface of the culture medium, there are zones of inhibition around the microcompresses (lack of bacterial growth), depending on the potency of the test against the bacterial species. They are directly proportional to the susceptibility of the germ in that the microbial growth inhibition zone is more extensive as the substance in the plant structure is more active. The results were expressed as average values obtained by performing the arithmetic mean of the diameters corresponding to the three tests [16].

Molecular docking technique

We used the Gaussian program suite at DFT/B3LYP/6-31G for antibiotic and poliphenol optimization. The X-ray crystal structure of the bacterial species was taken from the Protein Data Bank (3Q8U code for *Staphylococcus aureus*, 3T88 code for *Escherichia coli*, 1AX4 code for *Proteus vulgaris*, 4LKD code for *Pseudomonas aeruginosa*, 5O79 code for *Klebsiella pneumoniae*). The molecular docking analysis was performed using the Autodock 4.2.6 software together with the AutoDockTools molecular viewer [17]. Autodock can calculate and visualize the best conformation ligand-receptor and estimate minimum bonding energy [18]. The preparation of receptor molecule involves adding polar hydrogens, computing the Gasteiger charge; the grid box was created using Autogrid 4 with 80×80×95 Å in x, y and z directions with 1 Å spacing. All the calculations were performed in vacuum. For the docking process we chose the Lamarckian genetic algorithm, with a population size of 150 and a number of 30 runs.

Results and discussions

From analysis of HPLC results we noticed that the tincture from the VF-f contains apigenol in the traces. Recent research report that apigenin represents another molecular

Table 1

INHIBITION DIAMETER AVERAGE OF BACTERIAL GROWTH

Test Product	*Staphylococcus aureus*	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
VM-f	24,3***	20,1***	18,1***	18,4***	22,3***
amoxicillin (control +)	31,5***	nt	nt	nt	nt
levofloxacin (control +)	nt	34,1***	nt	nt	nt
amikacin (control +)	nt	nt	33,5***	nt	nt
ceftazidime (control +)	nt	nt	nt	28,6***	nt
cefotaxime (control +)	nt	nt	nt	nt	35,9***
ethyl alcohol 70 °	16.1*	7,5*	0*	0*	0*

*resistant **intermediary ***sensible; nt-not tested

Ligands	*Staphylococcus aureus*	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*
VM-f (apigenol)	-4.77	-5.27	-5.43	-5.34	-5.67
amoxicillin (control +)	-5.66	-	-	-	-
levofloxacin (control +)	-	-7.05	-	-	-
amikacin (control +)	-	-	-7.88	-	-
ceftazidime (control +)	-	-	-	-6.98	-
cefotaxime (control +)	-	-	-	-	-7.77
ethyl alcohol 70 °	-2.49	-2.56	-2.75	-0.6	-0.2

Table 2
FREE BINDING ENERGY LIGAND-RECEPTOR (kcal/mol)

class of natural antibiotic, being active even against quinolone-resistant bacteria [19].

The vegetal extract derived from the leaves of bilberry have therapeutic efficacy against all strains of bacterial microorganisms, which can be classified as susceptible to the tincture. However, the plant extract has therapeutic efficacy inferior to the antibiotic of choice for all bacteria. Ethyl alcohol (negative control) has a slight antibacterial effect on *Staphylococcus aureus* and *Escherichia coli*, the bacteria being intermediate-sensitive to it. Ethyl alcohol is ineffective on *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* (table 1).

Germs are classified as susceptible if the diameter of the inhibition zone is greater than 19 mm for *Staphylococcus aureus*, higher than 17 mm for *Escherichia coli* and *Proteus vulgaris*, 18 mm for *Pseudomonas aeruginosa* and 23 mm for *Klebsiella pneumoniae*.

Molecular modeling programs help researchers in all fields to find new terapeutic targets for the development of new drugs [20, 21]. Molecular docking technique helps us to understand ligand-receptor interaction, to know binding energy and the stability of the complex [22-23].

From the docking analysis results, it can be seen that the apigenol-bacteria binding energy complex is greater than the antibiotic-bacteria. This suggests a high stability of the complex antibiotic-bacteria, being more stable and more favorably energetic.

Apigenol-GABAA complex has a small binding energy, which explains the antibacterial effectiveness of the polyphenolic compound.The theoretical results are in good agreement with the experimentally antibiogram. Alcohol is intermediate active against *Staphylococcus aureus* and inactive on other bacterial strains (table 2).

Conclusions

Bilberry contains many components, but anthocyanins, the phenolic compounds that give berries their red, blue, and purple colors, have been found to have a wide range of health-related properties, including antioxidant, antitumorigenic, anti-inflammatory, hypoglycemic, and antimicrobial effects.

Apigenol is recognized for the antibacterial effect, VM-f containing apigenol in traces.

The vegetal extract derived from the leaves of bilberry have therapeutic efficacy against all strains of bacterial microorganisms which can be classified as susceptible to the tincture.

The theoretical results are in good agreement with the experimentally antibiogram, the complex antibiotic-bacteria, being more stable than apigenol-bacteria. These plant extracts could be a potential source of new antibacterial agents.

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