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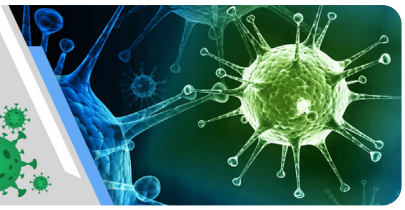

Multiple natural approaches of *Salix alba*

Marina Andrei[1], Amalia Ștefaniu[2], Nicoleta Ene[2], Lucia Pintilie[2], Nicoleta Radu[3, 4], Andrei Justinian Tomescu[5] and Anca Daniela Raiciu[*, 5, 6]

Abstract

The Willows, belonging to the genus Salix, encompass a vast array of species and hybrids, totaling between 330 to 500 species along with approximately 200 hybrids. These plants are found across continents such as Africa, North America, Europe, and Asia, exhibiting diverse forms ranging from trees to shrubs and prostrate plants. Traditionally, Willows have been utilized in folk medicine due to their rich reservoir of biologically active compounds, with salicin being a notable example, serving as a prodrug for salicylic acid. The chemical composition of Willows is highly diverse, with 322 secondary metabolites identified thus far. These include various classes such as flavonoids (94 types encompassing flavonols, flavones, flavanones, isoflavones, flavan-3-ols like catechins and procyanidins, chalcones, dihydrochalcone, anthocyanins, and dihydroflavonols), phenolic glycosides (76), organic acids (28), non-phenolic glycosides (17), sterols, and terpenes (17), simple phenolics (13), lignans (7), as well as volatiles and fatty acids (69). This rich chemical diversity underpins the various pharmacological activities exhibited by Willows. The pharmacological properties attributed to Willows are extensive and include analgesic, anti-inflammatory, antioxidant, anticancer, cytotoxic, antidiabetic, antimicrobial, antiobesity, neuroprotective, and hepatoprotective effects. These properties make Willows a subject of considerable interest in the fields of medicine and pharmacology. In summary, Willows represent a valuable natural resource with a diverse chemical composition and a wide range of pharmacological activities. This study aimed to enhance our comprehension of the medicinal characteristics of willow bark extract, focusing on its antimicrobial properties. This investigation holds significance in validating the traditional usage of willow bark and investigating its potential utility in contemporary medicine.

Keywords: Antimicrobial activity; *Salix alba*; Molecular docking

Introduction

The use of plants for medicinal purposes has indeed been a practice dating back to ancient civilizations. Plants contain a myriad of bioactive compounds that have been utilized by humans for treating various ailments and promoting health. With the emergence of pharmaceutical chemistry in the 19th century, the exploration of plant-derived substances for drug development intensified [1, 2]. One notable example of a plant with medicinal properties is white willow (Salix alba L.), a member of the genus *Salix* and the family *Salicaceae*. Willows are a diverse group of plants, ranging from small shrubs to towering trees, with white willow typically falling on the smaller end of the spectrum. White willow, also known as salicin willow, has a long history of use for

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its health benefits, spanning thousands of years. *Salicaceae*, commonly known as the Willow and Poplar family, has undergone taxonomic revisions to encompass additional genera beyond its traditional members, *Populus* (poplar) and *Salix* (willow). While traditionally limited to Northern temperate regions and characterized by the presence of catkins, the family now includes many tropical members from the Flacourtiaceae family, which do not produce catkins [3, 4]. This expansion has increased the family's diversity to approximately 56 genera and 1,220 species, as of 2016. Members of the *Salicaceae* family are renowned for their rapid growth, with many species being trees or shrubs. They hold significant economic value due to their versatility:

Salicaceae species are cultivated for their wood, which is used in various applications such as construction, furniture making, and woodworking.

The fast-growing nature of these trees makes them suitable for paper production, contributing to the pulp and paper industry.

The flexible twigs of willow species are utilized in crafts such as basket weaving, caning, and manufacturing woven fences and lattices.

Historically, *Salicaceae* species have been employed for making items like fences, snowshoes, arrow shafts, fish traps, whistles, nets, and rope.

They serve as a source of renewable energy, providing biomass fuel for heating and power generation.

Many *Salicaceae* species are cultivated for their aesthetic appeal in gardens and landscapes.

These plants play a role in environmental enhancement by aiding in soil erosion control. Their extensive root systems help stabilize soil in areas prone to erosion, making them valuable for land reclamation and restoration projects.

The active compound in white willow, salicin, has been recognized for its anti-inflammatory and analgesic properties. It is chemically similar to aspirin and serves as a precursor to the synthesis of acetylsalicylic acid, the active ingredient in aspirin. Due to its ability to alleviate pain and reduce inflammation, white willow has been traditionally employed to treat conditions such as headaches, muscle pain, and arthritis. Research conducted on white willow has provided insights into its pharmacological effects and potential therapeutic applications. Studies have suggested its efficacy in managing various forms of pain and inflammation, often with fewer side effects compared to synthetic alternatives.

Overall, white willow exemplifies the rich tradition of utilizing plant-derived remedies for healing purposes, demonstrating the ongoing relevance of natural products in modern healthcare practices [5, 6]. White willow (*Salix alba*) indeed has a rich history of medicinal use dating back thousands of years, as you've outlined. The use of its bark, containing salicin, for pain **relief** and inflammation management was widespread across various ancient civilizations, indicating its efficacy and value in traditional medicine. The mention of *Hippocrates* recommending chewing willow bark and using willow leaves for childbirth pain demonstrates the recognition of its analgesic properties since ancient times. Furthermore, its use in treating a variety of ailments such as rheumatic pain, back pain, toothache, menstrual cramps, sore throat, fever, and headache highlights its versatility in addressing different types of pain and inflammatory conditions. Modern research has also validated many of these traditional uses of white willow bark. It's recognized for its anti-inflammatory and analgesic effects, attributed mainly to salicin's conversion to salicylic acid in the body, which shares pharmacological similarities with aspirin. However, it's essential to note that while white willow bark can offer relief for various conditions, it may not be suitable for everyone, particularly those with certain medical conditions or allergies. As with any herbal remedy, it's advisable to consult with a healthcare professional before use, especially if you're pregnant, nursing, or taking other medications [7,8]. The study conducted aimed to explore various aspects of willow bark's medicinal properties. Specifically, they investigated the antimicrobial activity of ethanol extract from *Salix alba* (white willow) bark. The antimicrobial activity assessment likely involved testing the extract against various microorganisms to determine its effectiveness in inhibiting their growth. This is particularly relevant given the historical use of willow bark in traditional medicine for treating infections. Additionally, determining the total phenolic content and antioxidant activity provides insights into the extract's chemical composition and potential health benefits. Phenolic compounds are known for their antioxidant properties, which play a crucial role in protecting cells from oxidative damage and reducing the risk of various diseases. Overall, this study aimed to contribute to the understanding of the medicinal properties of willow bark extract, particularly in terms of its antimicrobial and antioxidant activities. Such research is essential for validating its traditional use and exploring its potential applications in modern medicine [6].

Materials and Methods

Phytochemistry

There are phytoconstituents or secondary metabolites found in the genus Salix, commonly known as willow trees. Flavonoids are a diverse group of plant secondary metabolites known for their antioxidant properties and various health benefits. They are commonly found in fruits, vegetables, and beverages such as tea and wine. Glycosides are compounds where a sugar molecule is attached to a non-sugar moiety.

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They can be further classified into phenolic glycosides (glycosides containing a phenolic group) and non-phenolic glycosides. Procyanidins are oligomeric flavonoids, also known as condensed tannins. They are found in various plants and are known for their antioxidant properties. Organic acids such as citric acid, malic acid, and tartaric acid are commonly found in plants. Their derivatives may include esters, salts, and other compounds formed from organic acids. Phenolic compounds are characterized by the presence of a phenol group.

They have diverse roles in plants, including defense against pathogens and environmental stressors. Sterols are a type of lipid that play structural roles in cell membranes. Terpenes are a large class of compounds derived from isoprene units, with diverse biological activities. Lignans are phenolic compounds found in plants, particularly in the cell walls. They have antioxidant and estrogenic properties. Volatile compounds are organic molecules with low molecular weights that evaporate easily at room temperature. Fatty acids are long-chain carboxylic acids commonly found in fats and oils [9].

Flavonoids

The distribution of various flavonoids across different species of the Salix genus, including their occurrence in different plant parts such as leaves, roots, and bark [10,11]. *Salix* species contain a wide variety of flavonoids, including flavones, flavonols, flavanones, dihydroflavonols, isoflavones, chalcones, dihydrochalcones, flavan-3-ols, and anthocyanins. The highest diversity of flavonoid classes was detected in leaves with rare occurrence in roots. Flavones, such as apigenin and its glycosides, are major constituents of *S. acutifolia*, *S. matsudana*, and *S. babylonica* leaves and roots. Chrysoeriol and its derivatives are major constituents of *S. babylonica*, *S. matsudana*, and *S. subserrata* leaves [12].

Acylated luteolin glucosides are characteristic of *S. gilgiana* leaves. Kaempferol and its derivatives are prominent in *S. bordensis*, *S. babylonica*, and *S. integra* × *S. suchowensis*. Angeloxyflavone and isoflavones are markers for *S. cheilophila* twigs. Sulfated flavanones and dihydroflavonols accumulate in *S. integra* × *S. suchowensis* young stems. The bark of willows contains the highest number of chalcones, catechins, procyanidins, and anthocyanins. Chalcones accumulate in the bark of *S. daphnoides*, *S. elbursensis*, *S. acutifolia*, and *S. rubra*. Catechins, epicatechin, and various procyanidins are major constituents of *S. sieboldiana* bark. Procyanidins are also significant in *S. daphnoides* bark. Anthocyanins are found in the bark of several Salix species, including *S. purpurea*, S. *alba, S. phylicifolia, S. nigricans, S. calodendron, S. viminalis, S. triandra*, and *S. amygdalina* [13] (Figure 1).

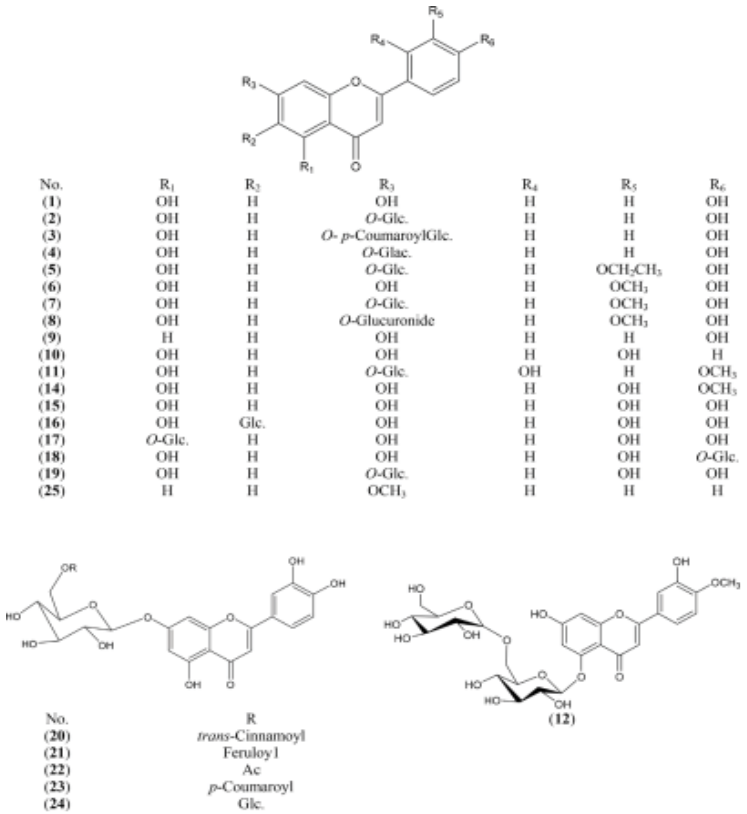

Figure 1: Structures of reported flavonoids from the genus Salix

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Phenolic Glycosides

It's important the classification and distribution of phenolic glycosides within the Salicaceae family, particularly focusing on the genus *Salix* [14]. These glycosides play important roles as secondary metabolites and can serve as taxonomic markers for different *Salix* species. The presence of specific glycosides like salicin, tremuloidin, and tremulacin, as well as others such as acmophyllin A and B, chaenomeloidin, cochinchiside, lasiandrin, leonuriside, and salicin-7-sulfate, can help distinguish between various *Salix* species [15,16].

Furthermore, the identification of certain glycosides, such as acutifoliside and 1,2-cyclohexanediol glycosides, serves as chemical markers for specific parts of Salix plants, like juvenile stems or twigs, aiding in their characterization and classification [17,18]. Overall, the presence and distribution of these phenolic glycosides provide valuable insights into the taxonomy and phytochemistry of *Salix* species [19] (Figure 2).

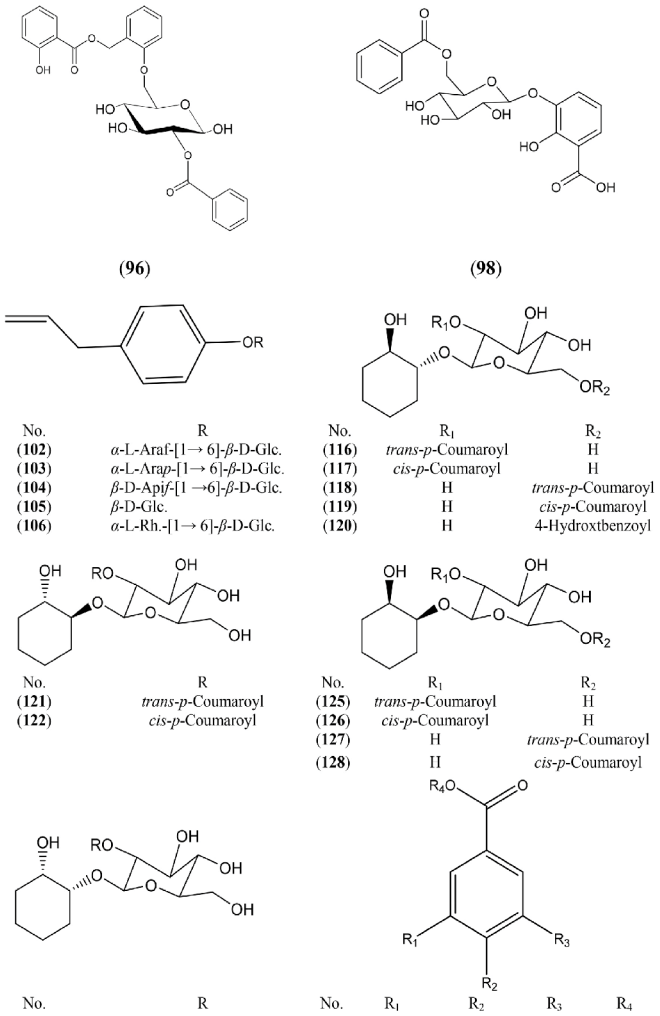
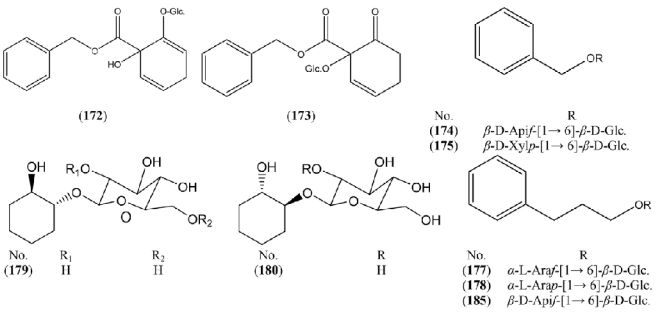


Figure 2: Structures of reported phenolic glycosides from genus *Salix*

Non-Phenolic Glycosides

In *S. triandra L.* x dasyclados Wimmer Wood, non-phenolic glycosides (compounds 172, 173, 174, 175, 176, 182–188) were identified as major constituents [19]. In *S. arbusculoides* Andersson twigs, compounds 170 and 171 are reported as major constituents [20]. Certain *Salix* species are characterized by the accumulation of 1,2-cyclohexanediol glycosides.



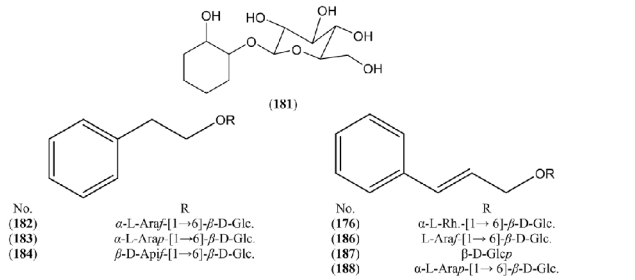


Figure 3: Structures of reported non-phenolic glycosides from genus *Salix*

Organic Acids

Phenolic acids are in various *Salix* species, which can occur in either free or esterified forms, such as benzyl, cinnamyl, or phenyl ethyl esters. These aromatic acids can be derivatives of benzoic or cinnamic acid. Benzoic acid derivatives: examples include p-hydroxybenzoic, p-anisic, gallic, salicylic, gentisic, vanillic, 2-amino-3-methoxy benzoic, and protocatechuic acids. Hydroxycinnamic acid derivatives: these include p-coumaric, caffeic, isoferuolic, and feruolic acids. Specific compounds and their occurrences in different *Salix* species are mentioned: *S. purpurea L.* and *S. alba L.* bark were found to contain the highest number of organic acids, including compounds 192–194, 198–200, and 214[21]. *S. tetrasperma* Roxb. flowers and bark contain compounds 197, 202, 203, 204, 205–206, 208, 209, and 215 [22].

Simple Phenolics

There are a variety of simple phenolic compounds found within the genus Salix, including phenolic acids and their derivatives. Accumulation of salicyl alcohol (compound

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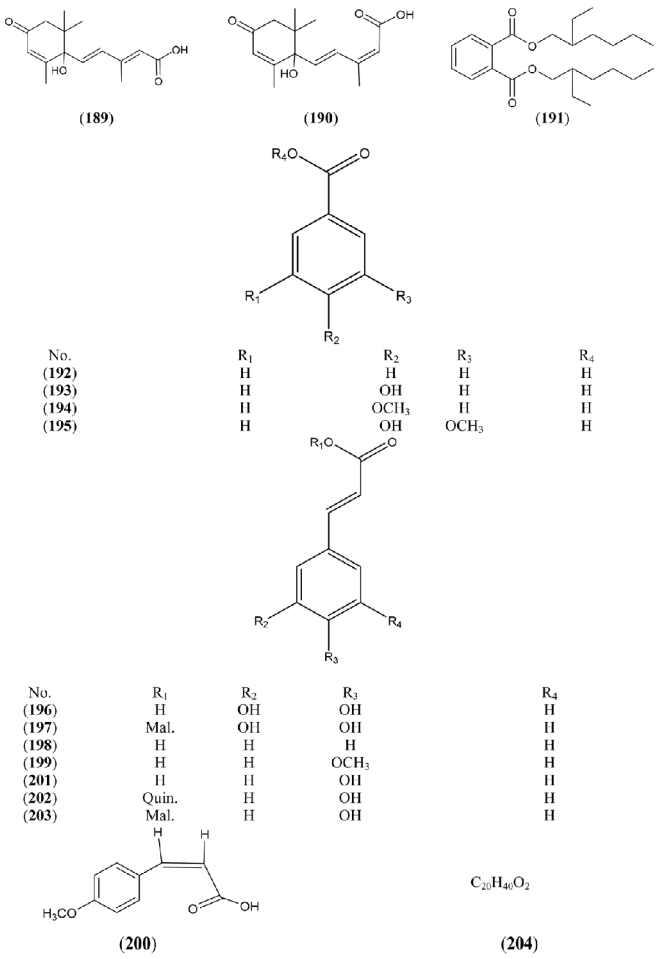

Figure 4: Structures of Reported Organic Acids From Genus Salix

228): this compound serves as the basic nucleus for salicinoids, identified in: *S. capensis* Thunb. Bark [23], S. acutifolia Willd. Bark [24], *S. subserrata* Willd. Bark [25] ,*S. caprea L.* inflorescence[26]. Accumulation of different simple phenolics in *S. caprea L.* wood: aucuparin (compound 218), methoxyaucuparin (compound 219), coniferyl alcohol (compound 221),p-coumaryl alcohol (compound 222),4,2′-dihydroxy-3,5-dimethoxybiphenyl (compound 223),sinapylaldehyde (compound 229),identified in S. caprea L. wood[27].

Sterols and Terpenes

Highest number of sterols and triterpenes are detected in: *S. cheilophila* C. K. Schneid. twigs: this species exhibited the highest number of sterols and triterpenes [28]. *S. tetrasperma* Roxb. bark, leaves, and flowers: significant amounts of sterols and triterpenes were found in different parts of this species [29]. *S. subserrata* Willd. leaves: this species also showed the presence of sterols and triterpenes [30]. *S. denticulate* aerial parts: detection of sterols and triterpenes was reported in the aerial parts of this species [31]. *S. babylonica L.* roots: sterols and triterpenes were found in the roots of this species

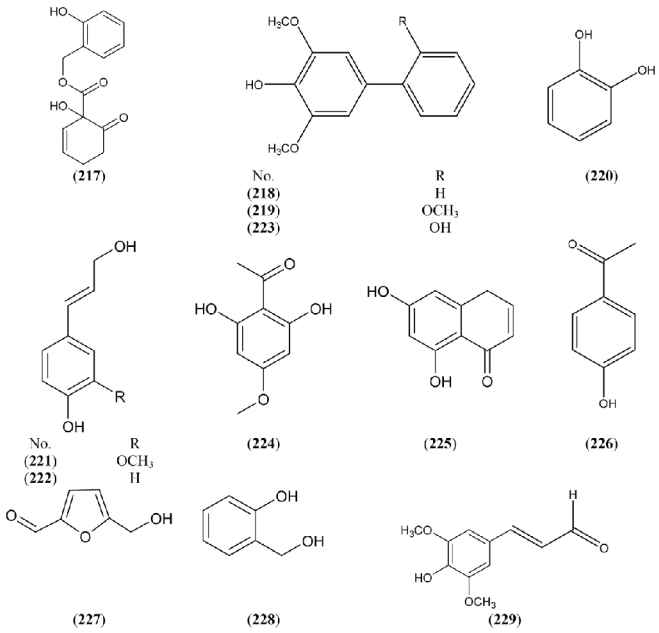

Figure 5: Structures of reported simple phenolics from genus *Salix*

[32]. *S. subserrata* Willd. bark and leaves: both bark and leaves of this species contained sterols and triterpenes [33]. Specific constituents found in S. cheilophila C. K. Schneid. twigs: Phytane and pimarane diterpene were identified as major constituents in the twigs of this species [28].

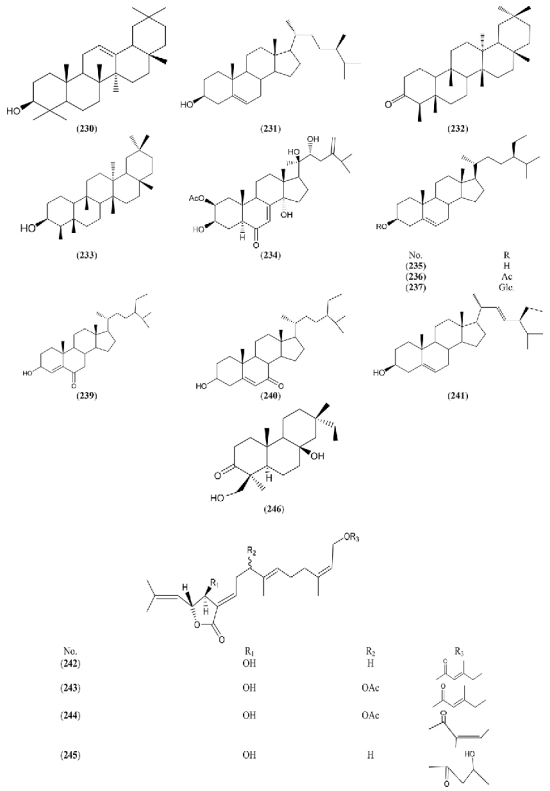

Figure 6: Structures of reported sterols and terpenes from genus *Salix*

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Lignans

The isolation of lignan derivatives from *Salix alba L.* bark, as well as the detection of various lignans in the biomass of five willow species cultivated in Quebec, Canada. Isolation from *S. alba L.* bark:sisymbrifolin, a lignan derivative (compound 247), was isolated from the bark of *Salix alba L.*[34].Detection in the biomass of five willow species in Quebec, Canada:pinoresinol (compound 248), lariciresinol (compound 249),secoisolariciresinol (compound 250),7-hydroxymatairesinol (compound 251),medioresinol (compound 252),lariciresinol-sesquilignan (compound 253). These lignans were detected in the biomass of five willow species cultivated in Quebec, Canada[35].

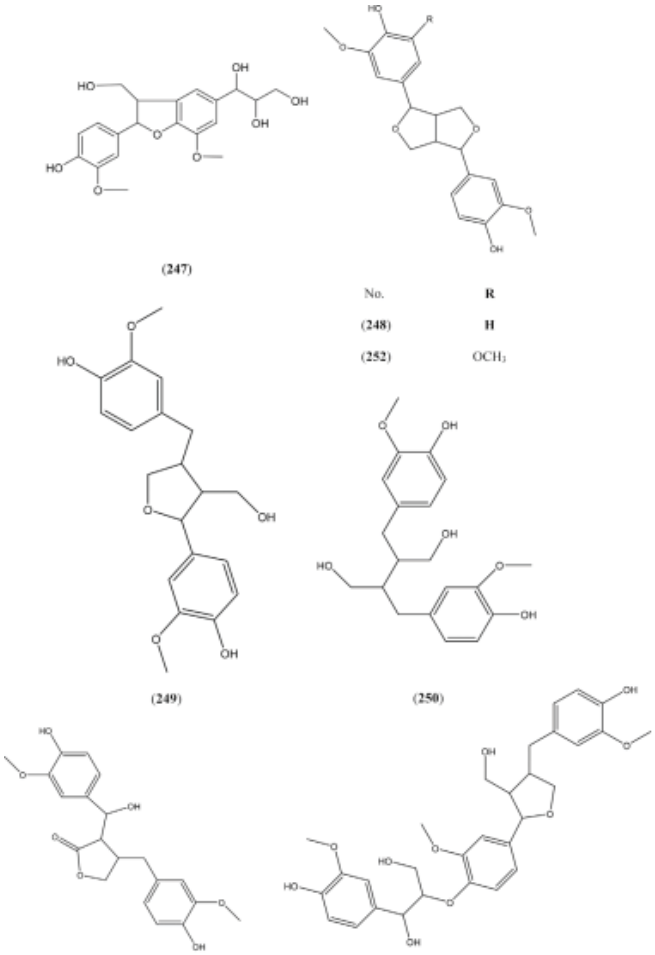

Figure 7: Structures of reported lignans from genus *Salix*

Volatiles

Terpenes (hemi-, mono-, and sesqui-terpenes) and non-terpene compounds (aliphatic and aromatic acids, their esters, carbonyl compounds, and hydrocarbons), in the genus Salix identified in the genus *Salix*:hemi-, mono-, and sesqui-terpenes,aliphatic and aromatic acids,esters,carbonyl compounds,hydrocarbons.There are highest percent of volatiles and fatty acids reported in certain parts of specific *Salix* species:*S. caprea L.* inflorescence: the highest percentage of volatiles and fatty acids was reported in the inflorescence of this species[26].Leaves of *S. egyptiaca L., S. babylonica L.*, and *S. alba L.*: high percentages of volatiles and fatty acids were reported in the leaves of these species[36].

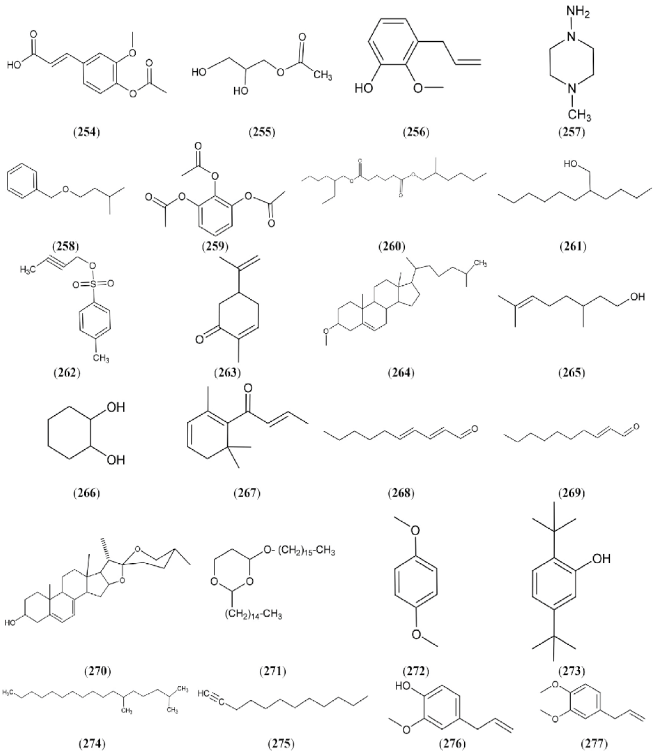

Figure 8: Structures of reported volatiles and fatty acids from genus Salix

Pharmacological Activity

Are well know the traditional use of various *Salix* species and their isolated com-pounds, such as salicylic acid and salicin, in folk medicine to treat various ailments in-cluding rheumatic diseases, back pain, toothache, headache, and menstrual cramps. Additionally, it mentions the diverse range of biological activities exhibited by *Salix* species and their compounds, including analgesic, antiinflammatory, antioxidant, anticancer, cytotoxic, antidiabetic, antimicrobial, anti-obesity, neuroprotective, and hepatoprotective activities.

Specifically, salicylic acid is noted for its effects on cyclooxygenases (COX I, II), which are enzymes crucial for the synthesis of prostaglandins, molecules involved in in-flammation and pain regulation[37].

Antiinflammatory Activity

Inflammation and its consequences: Inflammation is a common response to various stimuli such as microbial infection and injury. While inflammation is essential for

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controlling infection and promoting tissue repair, unresolved inflammation can contribute to the pathogenesis of diseases like atherosclerosis, obesity, cancer, and inflammatory bowel disease[38].

Anti-inflammatory effects of *Salix* extracts:

S. tetrasperma Roxb.: hydroalcoholic extract of S. tetrasperma Roxb. demonstrated an-ti-inflammatory effects in a rat paw edema model induced by carrageenan. It inhibited cyclooxygenases (COX-1, COX-2), lipoxygenase (LOX), and reduced levels of tumor necro-sis factor-alpha (TNF-a) and nuclear factor kappa B (NF-κB)[39]. S. canariensis: oral administration of *S. canariensis* extract showed dose-dependent anti-inflammatory activities, attributed to pentacyclic triterpenes and polyphenolics[40]. *S. caprea L.*: identified as a potent cyclooxygenase inhibitor[41].*S. subserrata Willd.* and *S. tetrasperma Roxb.*: exhibited anti-inflammatory effects against carrageenan-induced hind paw edema due to the presence of phenolic glycosides, especially salicin, as well as flavonoids like luteolin, quercetin, and rutin[42]. *S. matsudana Koidz.*: Methanol extract of S. matsudana Koidz. leaves showed inhibi-tory activities against cyclooxygenases (COX-1 and COX-2) due to the presence of matsu-done, luteolin 7-O-glucoside, and 4′,7-dihydroxyflavone[43].These findings suggest that *Salix* extracts possess significant anti-inflammatory properties, potentially attributed to various bioactive compounds present in different species.

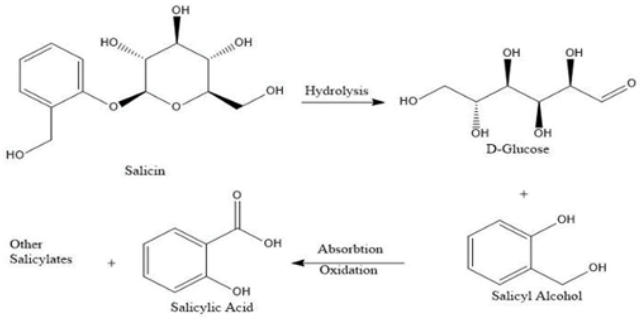

Figure 9: Conversion of Salicin→Saligenin→Salicylic Acid[52]

HPLC Method

Salicin, found in willow bark, has garnered attention for its therapeutic properties, particularly its anti-inflammatory effects. Single drug therapyis focusing on the use of a single medication for treating a particular condition, has a long history dating back to ancient times[44,45]. It's widely accepted by physicians today for various diseases.With the increased use of herbal remedies alongside conventional medications, understanding interactions between herbs and drugs has become crucial in clinical practice to ensure safety and efficacy.Developing a marker profile is vital for quality control and scientific validation of single drugs. This ensures consistency in composition and potency, aiding in reliable therapeutic outcomes.It's described as the metabolic precursor

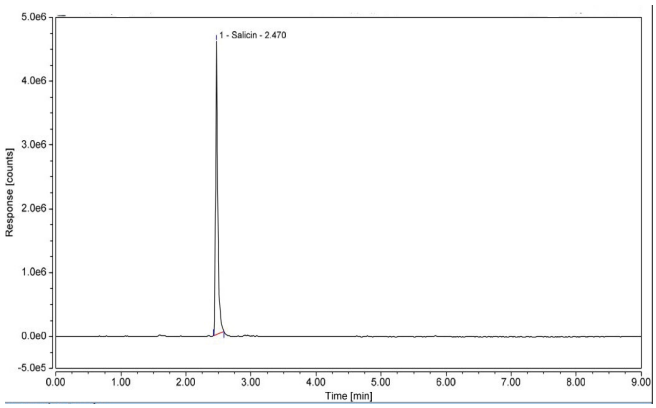

Figure 10: Concentration of Salicin from Salix alba bark from Olt district (Romania)

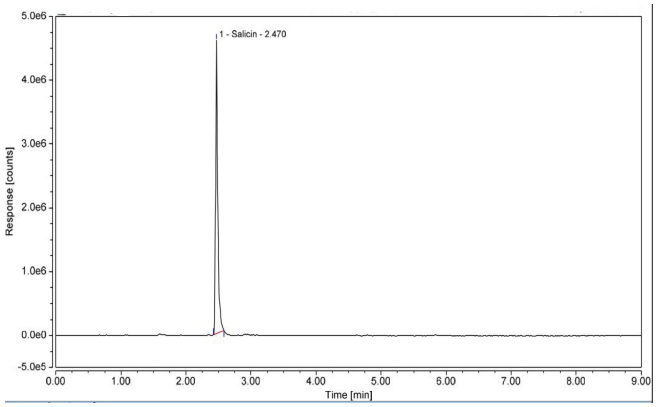

Figure 11: Concentration of Salicin from Salix babylonica bark from Olt district(Romania)

of salicylic acid, possessing anti-inflammatory properties. Salicin is an alcoholic beta-glycoside containing D-glucose, found in willow barks, particularly in *Salix alba L* (White Willow). It's noted as a weaker forerunner of aspirin[46]. Willow bark contains various chemical compounds, including glycosides (such as salicin), tannins, aromatic aldehydes and acids, salicyl alcohol (saligenin), and flavonoids[47-51]. These constituents contribute to its pharmacological effects. Salicin exhibits antipyretic and analgesic effects, making it useful in treating fever and conditions like arthritis[46]. Its similarity to aspirin in action.(Figure 9).

Methods

HPLC was performed using an Agilent EZChrom Elite system equipped with a DAD detector and a HiCHROM LiChrosorb 100 RP-18 column, 10 µm particle (4.6 x 250 mm).Extraction of salicylic derivatives: for the analysis of the samples represented by branches of willow.The sample (1 g) was extracted with 8 mL of a solution consisting of a part of

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4.2 g/L NaOH solution and a part of methanol. The mixture was stirred at 60°C at reflux for 60 min. After cooling, 0.4 mL of 10 g/L HCl solution was added and the mixture was centrifuged at 9000 rpm for 5 minutes. The supernatant was diluted to 10 mL with a mixture of equal volumes of methanol and high purity water. Before HPLC analysis, the samples were filtered with a PA filters.

Salicin derivatives are the main constituents of willow bark and can be quantified as salicin equivalents. According to the Monograph of the European Pharmacopoeia[3] and the Evaluation Report of the European Medicines Agency [52], the content of salicin derivatives in the bark of different species of willow varies from 0.5 to 10%. However, quality standards involve at least 1.5%.Although willow bark remains the main source of salicin.

Extraction method for a aqueous extracts

We prepare the aqueous extracts from *Salix alba* bark.

Preparation of stock aqueous extracts:10 grams of air-dried and milled plant material are soaked in 100 ml of distilled water, resulting in a concentration of 10% (w/v).The soaking occurs at room temperature (°C) for 24 hours with occasional shaking to facilitate the extraction process.

Filtration and centrifugation:after 24 hours of soaking, the mixtures are filtered through two layers of cheesecloth to remove solid particulate materials.The filtered mixtures are then centrifuged for 20 minutes at 10,000 rpm. This step helps to further remove any remaining particulate matter and debris, resulting in purified extracts.

Adjustment of pH:the purified extracts are adjusted to a pH of 6.8 using 1.0 M HCl (hydrochloric acid). pH adjustment is often done to optimize the stability and solubility of bioactive compounds present in the extract.

Storage:finally, the adjusted extracts are stored in the refrigerator at 4°C for future use. Storing the extracts at low temperature helps to preserve the stability and integrity of the bioactive compounds present in the extract.This method ensures the extraction of water-soluble compounds from the plant material and results in purified aqueous extracts suitable for further analysis or use in various applications such as pharmaceuticals, cosmetics, or food products.

Antimicrobial Assay

The Kirby-Bauer diffusometric method suitable for natural plant extracts was used to analyse the antimicrobial activity of the willow extract. This method is qualitative and allows the evaluation of the antimicrobial activity of the tested products based on the diameter of the inhibition surfaces for microbial growth. The antimicrobial method is based on the following principles: Stainless steel cylinders are used, which are placed on the surface of a specific growth medium inoculated with the microbial suspension to be tested (medium CaSoA - for the activation of bacterial strains). The tested extract is used in the cylinders. After incubation, the appearance of an inhibition zone of microbial growth was observed, which proves the sensitivity of the tested culture and the special properties of diffusion in the environment of the tested substance.The level of microbial activity was classified according to the diameter values of the inhibition zones according to the European Pharmacopoeia. The microbial strains selected for the test were *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538. The antimicrobial activity test was performed in triplicate.

Procedure for molecular docking

Molecular docking simulatuons were realized using CLC Drug Discovery Workbench (QIAGEN, Aarhus, Denmark) and MOLEGRO Virtual Docker software (Molexus IVS, Odder, Denmark), respectively. The docking protocol [53] include the mandatoy steps: ligand preparation, target preparation, choice of the suitable binding site, docking simulations for the co-crystallized ligand and for the ligand under investigation, salicin. Validation of the docking procedure was previosly realized in our published paper [54]. Simulations were carried out on the crystal structure of Escherichia coli Topoisomerase IV ParE 24kDa subunit, PDB ID: 1S14 [55]; on the twinned 3.35A structure of S. aureus Gyrase complex with Ciprofloxacin and DNA, PDB ID: 2XCT [Bax, B.D. 2010], and on the Candida albicans dihydrofolate reductase, PDB ID: 1AI9 [56]. For all cases, the native ligands' pose and interactins were firstly validated and the comparison for the docking score of the salicin was made.

Results

After the specific incubation period of 72 hours at 35°C, the results were analyzed. Bacterial development and the inhibition zone of the test extract were observed on the culture medium plates.

The willow plant extract analyzed showed moderate antimicrobial activity and had an inhibition zone diameter of 17 mm for *Escherichia coli* ATCC 8739 and 15.5 mm for Staphylococcus aureus ATCC 6538.

Experimental antimicrobial activity results

After the specific incubation period of 72 hours at 35°C, the results were analyzed. Bacterial development and the inhibition zone of the test extract were observed on the culture medium plates.The willow plant extract analyzed showed moderate antimicrobial activity and had an inhibition zone diameter of 17 mm for *Escherichia coli* ATCC 8739 and 15.5 mm for *Staphylococcus aureus* ATCC 6538.

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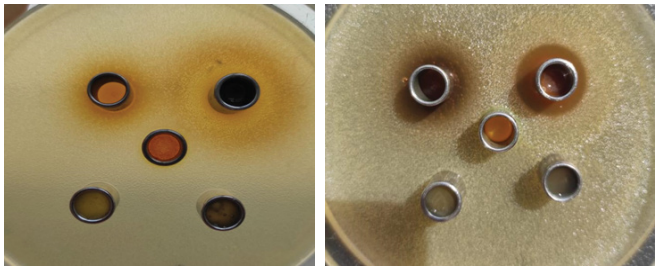

Figure 12: Zone of inhibition for the extract tested on *Escherichia coli* ATCC 8739 (right – top) Zone of inhibition for the extract tested on *Staphylococcus aureus* ATCC 6538 (right - top)

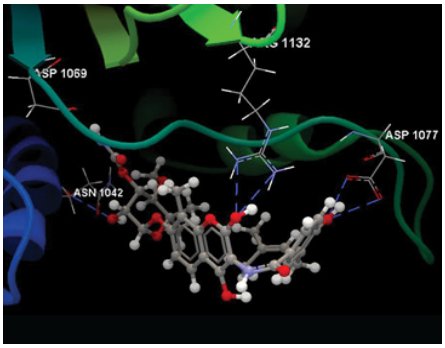

Figure 13: Hydrogen bonds between receptor, amino acids residues from 1S14 salicin co-crystallized ligand (novobiocin) receptor, obtained using CLC software

Molecular docking studies results

Results on *Escherichia coli simulations*

Figure 13 illustrates the interactions occurring between the native ligand, novobiocin and the amino acid residues from the active binding site of the Topoisomerase IV fragment from Escherichia Coli, pose obtained using CLC software. By comparison, in Figure 14, are given the image of the binging salicin in the same binding site and its hydrogen bonds formed with amino acids residues able to interact with its structure (SER 1043, ASP 1069, THR 1163, GLU 1046, ARG 1072, GLY 1073), forming in total 10 hydrogen bonds, as listed in Table 1.

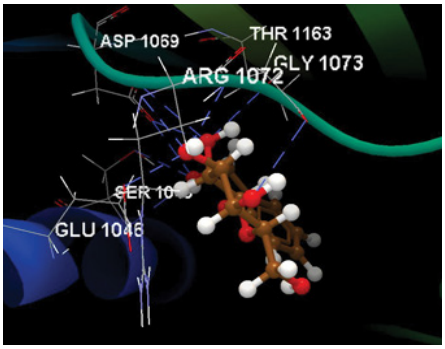

Figure 14: Hydrogen bonds between and amino acids residues from 1S14 obtained using CLC software

The Table 1 lists the length of hydrogen bond interactions formed by the investigated ligands, the co-crystallized novobiocin and salicin, respectively, within the active binding site of 1S14 receptor, along with the surrounding amino acids group interactions and the results of docking simulation in terms of docking score and the root mean square devia-tion (RMSD).

Table 1. The list of intermolecular interactions between the ligand molecules docked with 1S14 (CLC software)

Ligand	Score	RMSD (Å)	Amino acids group interaction	Hydrogen bond	Bond Length (Å)
NOV	-51.89	0.77	ASP 1077, ARG 1132, PRO 1075, ALA 1086, MET 1074, ARG 1072, GLY 1073, THR 1163, ILE 1090, ASP 1069, GLY 1071, GLU 1046, ASN 1042, SER 1043, ASP 1045	O sp^3 (O3) – O sp^2 ASP 1077 O sp^3 (O3) – O sp^2 ASP 1077 O sp^3 (O11) – N sp^2 ARG 1132 O sp^3 (O11) – N sp^2 ARG 1132 N sp^3(N1) – O sp^2 ASP 1069 O sp^3 (O6) – O sp^2 ASN 1042	3.122 2.686 3.152 3.106 2.645 2.730
Salicin	-49.75	0.06	LEU 1091, ILE 1090, VAL 1039, VAL 1067, ASN 1042, VAL 1165, SER 1043, SER 1164, ASP 1069, GLU 1046, THR 1163, MET 1074, GLY 1162, GLY 1071, GLY 1073, ARG 1072, PRO 1075, ARG 1132.	O sp^3 (O7) – O sp^3 SER 1043 O sp^3 (O7) – N sp^2 SER 1043 O sp^3 (O7) – O sp^2 ASP 1069 O sp^3 (O5) – O sp^2 ASP 1069 O sp^3 (O5) – O sp^2 ASP 1069 O sp^3 (O5) – O sp^3 THR 1163 O sp^3 (O3) – O sp^2 GLU 1046 O sp^3 (O3) – N sp^2 ARG 1072 O sp^3 (O3) – N sp^2 GLY 1073 O sp^3 (O4) – O sp^2 GLY 1073	2.678 3.200 3.100 2.824 3.286 2.656 2.660 2.950 2.780 3.044

Interactions obtained following the simulations carried out on the native ligand Novobi-ocin, using Molegro software are depicted in Figure 13.

Citation: Marina Andrei, Amalia Ștefaniu, Nicoleta Ene, Lucia Pintilie, Nicoleta Radu, Andrei Justinian Tomescu and Anca Daniela Raiciu. Multiple natural approaches of Salix alba. Archives of Microbiology and Immunology. 8 (2024): 410-427.

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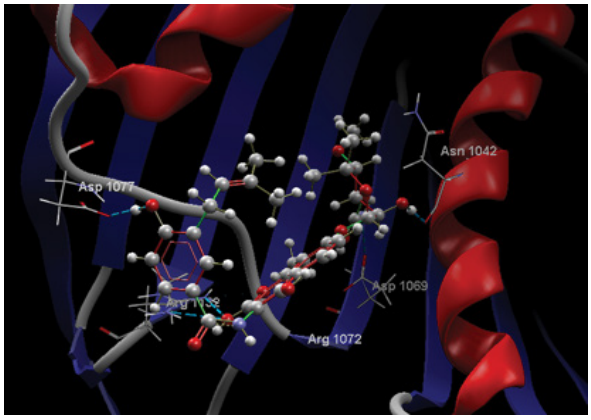

Figure 15: Hydrogen bonds between co-crystallized ligand (Novobiocin) and amino acids residues from 1S14 receptor, obtained with Molegro software

More detailed than the Figure 15, in Table 2 are shown the docking score and the type of interactions (steric interactions and hydrogen bond interactions, respectively) observed using Molegro software for novobiocin, compared with salicin.

Table 2. The list of intermolecular interactions between the ligand molecules docked with 1S14 (Molegro software)

Ligand	Score	Group interaction	Hydrogen bond	Bond Length, (Å)	Steric interactions	Distance (Å)
NOV	-100.89	ALA 1086, ARG 1072, ARG 1132, ASN 1042, ASP 1045, ASP 1069, ASP 1077, GLU 1046, GLY 1073, ILE 1090, MET 1074, PRO 1075, SER 1043, THR1163	O sp^3 (O1) – O sp^2 ASN 1042 N sp^2(N1) – O sp^2 ASP 1069 O sp^3 (O3) – O sp^3 ASP 1077 O sp^3 (O11) – N sp^2 ARG 1132 O sp^2 (O11) – N sp^2 ARG 1132	2.729 2.645 2.685 3.105 3.152	C sp^2 (C18) – N sp^2 ARG 1077 C sp^2 (C8) – N sp^2 ARG 1072	3.17 3.16
Salicin	-98.29	ARG 1072, ARG 1132, ASN 1042, ASP 1069, GLU 1046, GLY 1071, GLY 1073, GLY 1162, ILE 1090, MET 1074, PRO 1075, SER 1043, THR 1163, VAL 1039, VAL 1067, VAL 1165	O sp^3 (O7) – O sp^2 VAL 1039 O sp^3 (O7) – O sp^3 SER 1043 O sp^3 (O5) – O sp^2 ASP 1069 O sp^3 (O5) – O sp^3 THR 1063 O sp^3 (O3) – N sp^2 GLY 1073 O sp^3 (O3) - N sp^2 ARG 1072 O sp^3 (O3) - O sp^2 GLU 1046 O sp^3 (O3) - O sp^2 GLY 1073	2.735 2.818 2.835 2.601 2.743 2.942 2.617 3.082	C sp^3 (C12) – C sp^3 MET 1074 O sp^3 (O5) – C sp^3 THR 1163 O sp^3 (O3) – C sp^3 GLU 1046 O sp^3 (O3) – C sp^2 GLU 1046	3.150 2.933 3.063 2.903

Results on *Staphylococcus aureus* simulations

In Figure 16, the hydrogen bonds formed by the co-crystallized ciprofloxacin, a fluoroquinolone structure, used as strong antibiotic to treat a number of bacterial infections, in its complex formed with *S. aureus* gyrase and DNA, given by 2XCT fragment. Figure 17 illustrates the hydrogen bonds formed by salicin with the same receptor, within the active binding site.

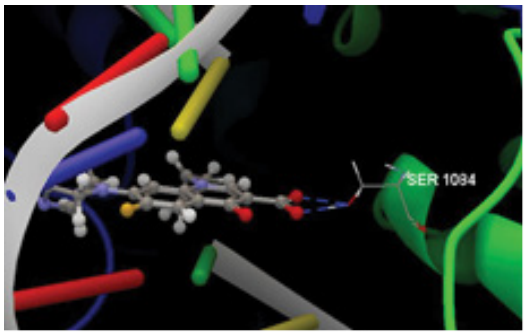

Figure 16: Hydrogen bonds between cocrystallized ligand (ciprofloxacin) and amino acids residues receptor from 2XCT receptor

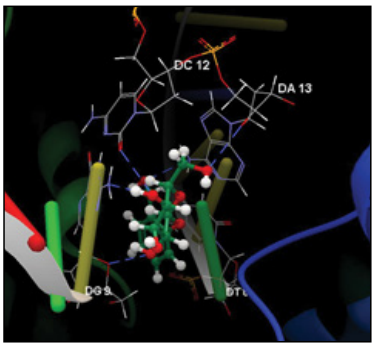

Figure 17: Hydrogen bonds between salicin and amino acids residues from 2XCT

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In Table 3 are given the results of docking simulations on 2XCT receptor for ciprofloxacin (CPF) and salicin, obtained by means of CLC software.

Table 3. The list of intermolecular interactions between the ligand molecules docked with 2XCT (CLC software)

Ligand	Score	RMSD	Group interaction	Hydrogen bond	Bond Length (Å)
CPF	-80.60	0.67	ASP 437, SER 1085, SER 1084, GLY 1082, GLY 459, LYS 460, ARG 458, GLU 477, DA 13:H, DC 12:H	O sp^2 (O1) – O sp^3 SER 1084:B O sp^2 (O2) – O sp^3 SER 1084:B	2.489 2.604
Salcin	-64.52	0.44	ASP 437, SER 1084, GLY 459, DT 10 :G, DG 9:G, DT 8:E, DG 7:E, DA 13:H, DC 12>H	O sp^3 (O7) – O sp^2 DT 8:E O sp^3 (O7) – N sp^2 DA 13:H O sp^3 (O5) – O sp^3 DG 9:G O sp^3 (O4) – O sp^3 DG 9:G O sp^3 (O4) – O sp^2 DC 12:H O sp^3 (O3) – O sp^3 DA 13:H	2.881 3.016 2.844 3.069 3.006 3.070

The results obtained by simulations using Molegro software, for CPF and salicin on the same receptor, S. aureus gyrase, are illustrated for ciprofloxacin in Figure 18, and for sa-licin in Figure 19, respectively. The details of interactions and score obtained with Molegro software are given in Table 4.

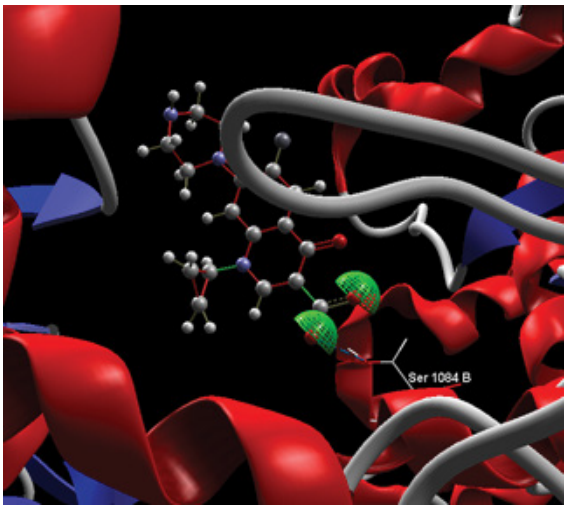

Figure 18: Hydrogen bonds between co-crystallized ligand (Ciprofloxacin) and amino acids residues from 2XCT receptor

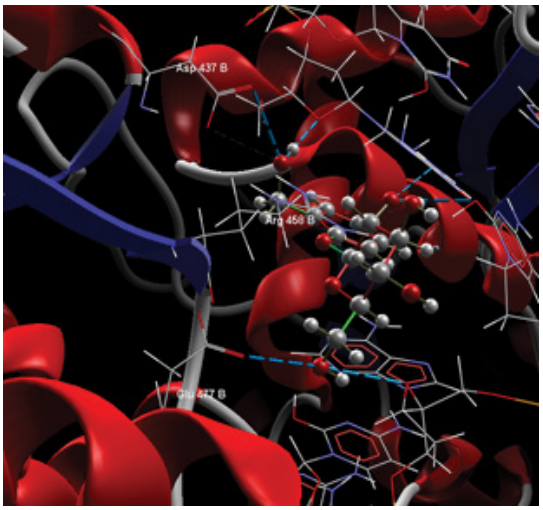

Figure 19: Hydrogen bonds between salicin and amino acids res-idues from 2XCT receptor

Table 4. The list of intermolecular interactions between the ligand molecules docked with 2XCT (MOLEGRO)

Ligand	Mol Dock Score	Molecule Contributions	Hydrogen bond	Bond Length (Å)	Electrostatic interactions / Steric interactions	Distance (Å)
CPF	-121.47	ARG 458:B, ASP 512:B, ASP 1083:B, GLU 1088:B, GLY 459:B, GLY 1082:B, LYS 417:B, LYS 460:B, LYS 1043:B, SER 1084:B, ARG 1122:D, LYS 1066:D, *DA…DA:G, **DT..DA:H.	O sp^2 (O1) – O sp^3 SER 1084:B	2.489	**Electrostatic interactions** O sp^2 (O1) – Mn O sp^2 (O2) – Mn	 2.089 4.409
					Steric interactions O sp^2 (O2) – C sp^3 from SER 1084:B C sp^2 (C3) – O sp^3 from SER 1084:B C sp^3 (C16) – C sp^3 from ARG 458:B	 3.024 2.932 3.152

Citation: Marina Andrei, Amalia Ștefaniu, Nicoleta Ene, Lucia Pintilie, Nicoleta Radu, Andrei Justinian Tomescu and Anca Daniela Raiciu. Multiple natural approaches of Salix alba. Archives of Microbiology and Immunology. 8 (2024): 410-427.

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Salicin	-94.21	ARG 458:B, ASN 476:B, ASP 437:B, GLU 477:B, GLY 459:B, *DA…DA:G, **DT..DA:H.	O sp^3 (O7) – O sp^3 ASP 437:B O sp^3 (O7) – O sp^2 ASP 437:B O sp^3 (O7) – O sp^3 *DA…DA:G O sp^3 (O5) – N sp^2 *DA…DA:G O sp^3 (O5) – O sp^3 **DT..DA:H O sp^3 (O3) – O sp^2 ARG 458:B O sp^3 (O6) – O sp^2 GLU 477:B O sp^3 (O6) – O sp^3 **DT..DA:H O sp^3 (O6) – N sp^2 **DT..DA:H	3.190 2.996 3.226 2.460 2.950 3.094 3.168 3.052 3.429	C sp^3 (C9) – N sp^2 ARG 458:B C sp^3 (C13) – O sp^2 GLU 477:B	2.695 3.219

* nucleic acids: chain G: DA-DT-DG-DG-DG-DC-DC-DC-DC-DC-DA;** nucleic acids: chain H: DT-DG-DG-DC-DC-DC-DC-DC-DA-DA-DA-DA

Results on *Candida albicans* simulations

The results of docking simulations on dihydrofolate reductase from *Candida albicans*, PDB ID: 1AI9 for the native ligand (NDP) and for salicin are illustrated in Figure 20 and Figure 21, respectively, representing their hydrogen bonds occurring within the active binding site of the receptor, with the amin acid residues. The details of these interactions and the docking score obtained using CLC software, are given in Table 5.

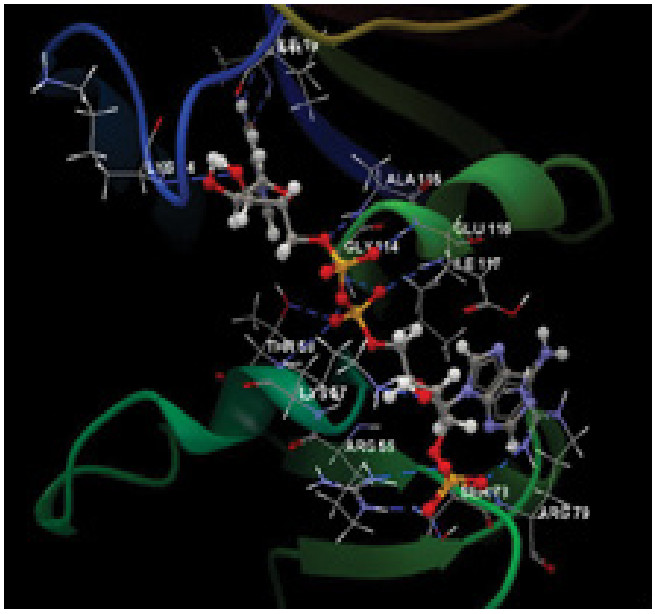

Figure 20: Hydrogen bonds between co-crystallized ligand (NDP) and amino acids residues from 1AI9 receptor

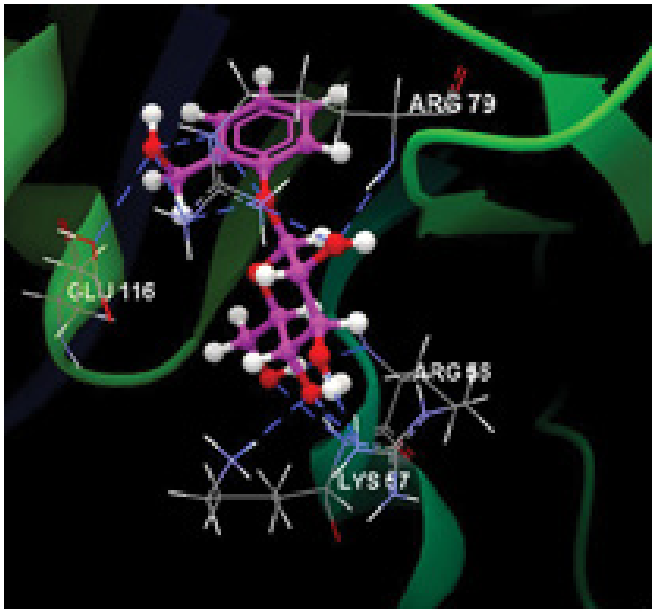

Figure 21: Hydrogen bonds between salicin and amino acids residues from 1AI9 receptor

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Table 5. The list of intermolecular interactions between the compounds docked with 1AI9 (CLC software)

Ligand	Score	RMSD (Å)	Group interaction	Hydrogen Bond	Bond Length (Å)
Co-crystallized NDP	-79.35	2.86	LEU 121, GLU 120, SER 95, TYR 118, GLU 116, ILE 117, SER 94, ARG 79, ILE 9, ALA 93, VAL 10, SER 78, LEU 77, ILE 112, GLY 113, MET 54, ALA 11, GLY 114, ILE 19, THR 147, GLY 20, TYR 21, PHE 36, ARG 56, LYS 57, THR 58, MET 25, GLY 23, PRO 26, LYS 24	N sp^2 (N7N) – O sp^2 ILE 112 O sp^3 (O2D) – O sp^2 ILE 19 O sp^3 (O4D) – N sp^2 ALA 115 O sp^3 (O5D) – N sp^2 ALA 115 O sp^2 (O2N) – O sp^3 THR 58 O sp^2 (O2N – N sp^2 THR 58 O sp^2 (O2A) – N sp^2 ALA 115 O sp^2 (O2A) – N sp^2 GLU 116 O sp^3 (O3B) – O sp^2 GLU 116 O sp^3 (O3B) – N sp^3 LYS 57 O sp^2 (O2X) – N sp^3 LYS 57 O sp^2 (O2X) – N sp^2 ARG 56 O sp^2 (O1X) – N sp^2 ARG 79 O sp^2 (O3X) – N sp^2 ARG 79 O sp^2 (O3X) – N sp^2 ARG 79 N sp^2 (N3A) – N sp^2 SER 78	3.069 2.649 3.335 3.394 2.977 3.194 3.111 2.733 2.892 2.924 2.772 2.365 2.698 2,984 3.061 3.286
Salicin	-57.52	0.26	SER 89, ARG 79, GLU 120, LEU 121, ILE 96, GLU 116, SER 95, SER 94, SER 78, SER 80, LEU 77, GLY 55, ALA 93, ARG 56, LYS 57, THR 58, MET 54	O sp^3 (O7) - O sp^3 GLU 116 O sp^3 (O7) – N sp^2 ARG 79 O sp^3 (O7) – N sp^2 ARG 79 O sp^3 (O5) – N sp^2 ARG 79 O sp^3 (O5) – N sp^2 ARG 79 O sp^3 (O3) – N sp^2 ARG 56 O sp^3 (O4) – N sp^2 ARG 56 O sp^3 (O4) – N sp^3 LYS 57 O sp^3 (O4) – N sp^2 LYS 57 O sp^3 (O6) – N sp^2 LYS 57 O sp^3 (O6) – N sp^2 ARG 56 O sp^3 (O2) – N sp^2 ARG 79 O sp^3 (O2) – N sp^2 ARG 79 O sp^3 (O2) – N sp^2 ARG 79	3.096 3.157 3.045 2.642 2.727 2.608 3.246 3.295 3.123 2.875 3.116 3.174 3.066 3.145

Figures 22 and 23, respectively, illustrate the hydrogen bonds formed by NDP and salicin, respectively within the binding site of 1AI9 receptor fragment, resulted during simulations with Molegro software. Furthermore, in Table 6, the results of docking studies with Molegro software for *Candida albicans*, are given as: hydrogen bonding, steric interactions and electrostatic interactions, along with their distance.

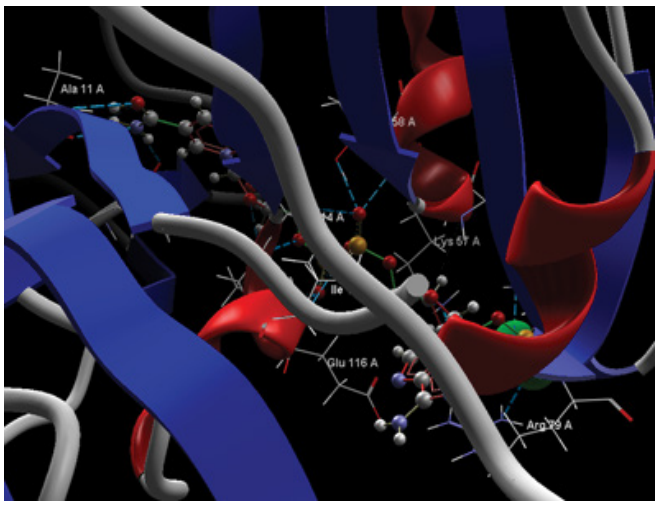

Figure 22: Hydrogen bonds between co-crystallized ligand (NDP) and amino acids residues from 1AI9.

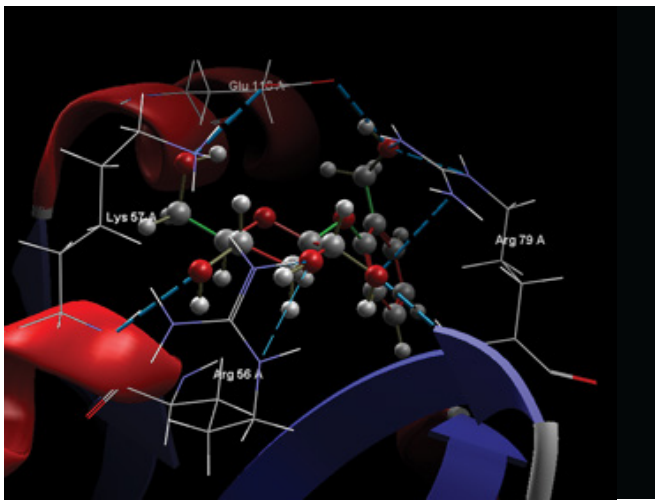

Figure 23: Hydrogen bonds between salicin and amino acids residues from receptor 1AI9 receptor.

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Table 6. The list of docking interactions between the ligand molecules and 1AI9 using MOLEGRO VIRTUAL DOCKER Software

Ligand	Mol Dock Score	Molecule contributions	Hydrogen bond	Bond Length (Å)	Electrostatic interactions / Steric interactions	Distance (Å)
Co-crystallized NDP	-155.24	ALA 11, ALA 12, ALA 93, ALA 115, ARG 49, ARG 56, ARG 67, ARG 72, ARG 79, ARG 191, ASP 87, ASP 146, GLU 32, GLU 60, GLU 82, GLU 84, GLU 97, GLU 116, GLU 120, GLY 20, GLY 23, GLY 55, GLY 113, GLY 114, HIS 92, ILE 19, ILE 112, ILE 117, LEU 77, LEU 121, LYS 3, LYS 14, LYS 22, LYS 24, LYS 31, LYS 37, LYS 45, LYS 57, LYS 65, LYS 150, LYS 158, LYS 178, LYS 192, MET 25, SER 78, SER 80, SER 94, SER 95, THR 58, THR 147, TRP 27, TYR 21, TYR 118, VAL 10	N sp^2 (N7N) – O sp^2 ILE 19 N sp^2 (N7N) – O sp^2 ALA 11 O sp^3 (O7N) – N sp^2 ALA 11 O sp^3 (O7N) – N sp^2 ALA 11 O sp^3 (O4D) – N sp^2 ALA 115 O sp^3 (O5D) – N sp^2 ALA 115 O sp^2 (O2A) – N sp^2 GLY 114 O sp^2 (O2A) – O sp^3 THR 58 O sp^2 (O2A) – N sp^2 THR 58 O sp^2 (O2N) – N sp^2 GLU 116 O sp^2 (O1A) – N sp^2 GLU 116 O sp^2 (O1A) – N sp^2 ILE 117 O sp^3 (O3B) – N sp^3 LYS 57 O sp^2 (O2X) – N sp^2 ARG 56 O sp^2 (O1X) – N sp^2 ARG 56 O sp^2 (O1X) – O sp^3 SER 78 O sp^2 (O3X) – N sp^2 ARG 79 O sp^2 (O3X) – N sp^2 ARG 79	3.204 2.887 3.093 3.514 3.437 2.930 2.813 2.565 3.218 3.109 3.405 3.302 2.585 3.199 2.893 2.455 2.757 2.545	**Electrostatic interactions** O sp^2 (O2X) – N sp^2 ARG 79 O sp^2 (O2X) – N sp^2 ARG 56 O sp^3 (O2X) – N sp^3 LYS 57 O sp^2 (O1X) – N sp^2 ARG 79 O sp^2 (O1X) – N sp^2 ARG 79 O sp^2 (O3X) – N sp^2 ARG 56 **Steric interactions** O sp^2 (O1A) – N sp^2 GLY 114 O sp^2 (O2A) – C sp^3 GLY 114 O sp^2 (O1X) – C sp^3 SER 78	3.716 3.199 4.399 2.545 4.247 3.681 3.109 3.181 3.153
Salicin	- 94.82	ARG 56, ARG 79, GLU 116, GLU 120, GLY 55, ILE 117, LEU 77, LEU 121, LYS 57, SER 78, SER 94, SER 95	O sp^3 (O7) - O sp^3 GLU 116 O sp^3 (O7) – N sp^2 ARG 79 O sp^3 (O7) – N sp^2 ARG 79 O sp^3 (O5) – N sp^2 ARG 79 O sp^3 (O5) – N sp^2 ARG 79 O sp^3 (O3) – N sp^2 ARG 56 O sp^3 (O4) – N sp^2 ARG 56 O sp^3 (O4) – N sp^2 LYS 57 O sp^3 (O4) – O sp^2 GLU 116	2.810 3.000 3.052 2.961 2.909 3.318 2.855 2.798 3.110	O sp^3 (O4) – C sp^3 LYS 57	2.939

Table 7. Calculated properties of salicin

Compounds	Atoms	Weight [Daltons]	Flexible bonds	Lipinski violations	Hydrogen donors	Hydrogen acceptors	Log P
Salicin	38	286.28	4	0	5	7	1.68

Table 7 lists the main parameters and descriptors tacked into account for assessment of the druglikeness of salicin according Lipinski's rule of five [57]: molecular mass, rotatable bonds count, number of hydrogen bond donors and acceptors, and the water-partition coefficient.

Discussion

After the specific incubation period of 72 hours at 35°C, the results were analyzed. Bacterial development and the inhibition zone of the test extract were observed on the culture medium plates. The willow plant extract analyzed showed moderate antimicrobi-al activity and had an inhibition zone diameter of 17 mm for Escherichia coli ATCC 8739 and 15.5 mm for Staphylococcus aureus ATCC 6538, as illustrated in Figure 12.

Regarding the antibacterial effect, the results obtained in this study are supported by the data presented in the specialized literature.

Some researches [58] tested the antimicrobial effect of bioproducts obtained from the bark of Salix sp. by extractions assisted by ultrasound, made with (alcohol + ethyl acetate). The results obtained by them showed that the bioproducts obtained exhibit antimicrobial effects against S. aureus, B. cereus, B. megatherium, S. fexeneri, B. anthracis, P. aeruginosa, S. boydi and E. coli, which obtain inhibition diameters located between (10-11) mm.

Other researchers [59] in the tests carried out with extracts obtained from the bark of Salix alba, report antimicrobial effects for E. coli, S. aureus and L. monocytes, for which they obtain the inhibition diameters situated between (0.5-3.08) mm.

Another researchers [60,61] in the investigations carried

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out with the crude bioprod-ucts obtained from the bark of Salix sp. by extraction with a mixture of methanol : water : acetic acid, have reported that these contain catechin, hydroxybenzoic acid, naringenin, piceol, quercitin, salicylic acid and their derivatives.

The tests carried out with these crude extracts reveal that they inhibit the develop-ment of S. aureus (the CFU number is reduced from 108 to 102 in the range of the brut ex-tract concentrations situated between (625-2500) µg/mL [60].

If the extraction is carried out in n-hexane, ethyl acetate or methanol, the resulting crude extracts inhibit the development of E. coli for which inhibited diameters between 7.8-11.7 mm are obtained) and S. aureus (the inhibition diameters obtained were situated between 7.2 and 14.5 mm). In the case of bioproducts obtained by extraction in an aqueous medi-um, the scientific literature reports antimicrobial effects for E. coli (inhibition diameter = 13 mm) and S. aureus (MIC = 0.38 mg/mL) [62,63].

In the tests carried out by some researchers [64] on aqueous extracts obtained from 6 species of Salix sp., an antimicrobial effect was put into evidence for S. aureus (MIC= 0.6-0.8) mg/mL. The following compounds were identified in the aqueous extracts obtained sug-ars (glucose, fructose, sucrose), salicin, picein and catechin [64].

In 2023, other studies [65,66] have reported strong antimicrobial effects for S. aureus and S. aureus MRSA [65], as well for C. albicans [57]. If the extractions from the bark of Salix alba is assisted by ultra-sound or microwaves, the brut extracts obtained have antibacterial effects for P. aeruginosa as well (inhibition diameters obtained = 8-19 mm).

Regarding the docking simulations results obtained for Escherichia coli, as show in Figures 13,14 and Table 1, using CLC software, the docking score for salicin is close to novo-biocin, suggesting strong interactions, as depicted by the formed hydrogen bonds among with ASP 1069 common amino acid residue. Although with a slightly lower score, salicin forms more hydrogen bonds, 10 compared to novobiocin which forms 6, suggesting a bet-ter complexation. The same situation is observed when performing studies on 1S14 re-ceptor fragment, using Molegro software, as given in Table 2. The score obtained for sali-cin is comparable with novobiocin (- 98.29 versus -100.89), but more interactions given by salicin. Accordingly, a strong antimicrobial effect of salicin against Escherichia coli can be expected.

Concerning the interactions occurring in complex with Staphylococcus aureus DNA gyrase, with both software, CLC and Molegro, respectively, as shown in Table 3 and its corresponding pictures (Figure 16 and Figure 17 , respectively), and Table 4, in accordance with Figures 18 and 19, respectively, very strong interactions are observed, both for salicin, and for ciprofloxacin. Additionally, ciprofloxacin shows steric interactions with Mn co-factor and steric interactions with SER1084 and ARG458, on chain B. As expected, the docking score for ciprofloxacin is greater in both cases, showing a stronger effect against Staphylococcus aureus than salicin. The magnitude of the docking score obtained for salicin, leads us to think that it could be used as an antimicrobial agent for less severe infections, with good therapeutic outcomes.

The results of docking simulations for Candida albicans, show lower docking score for salicin than for the native ligand, thus suggesting a weaker affinity of salicin and consequently, poor antimicrobial activity against this patogen.

Conclusion

The tests carried out with the extracts of Salix sp. obtained showed that raw bio-prep-arations have antimicrobial effects for microorganisms such as S. aureus, E. coli and C. albicans, the obtained results being supported by the current scientific literature. Overall, the antimicrobial potency and druglikeness of salicin (no deviation from Lipinski's rule of five (as shown in Table 7), entitles us not to neglect its therapeutical potential alone and, additionally, as core structure for designing new compounds with better properties.

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Not applicable.

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