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Antimicrobial Properties of *Quercus ilex* L. Proanthocyanidin Dimers and Simple Phenolics: Evaluation of Their Synergistic Activity with Conventional Antimicrobials and Prediction of Their Pharmacokinetic Profile

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Supporting Information

ABSTRACT: The antibacterial and antifungal activities of an ample number of phenolic compounds isolated from *Quercus ilex* leaves, belonging to the classes of flavonoids, proanthocyanidins, and phenolic acids, are discussed. The isolation of A type proanthocyanidin, (+)-epigallocatechin-(2β→O→7, 4β→8)-(+)-catechin (**1**) is reported for the first time. Its structure was established by means of highfield NMR (correlation spectroscopy, heteronuclear single quantum correlation, heteronuclear multiple bond correlation, and rotating frame Overhauser effect spectroscopy) and MS spectral analyses, while its absolute configuration was determined by circular dichroism measurements. The isolated compounds were tested for their antimicrobial effects against eight human bacterial species and 14 fungal species. In a second step, the most potent compounds were tested in combination with the conventional fungicides, bifonazole and ketoconazole, to evaluate possible synergistic effects. Results showed that proanthocyanidins **3** and **4** when combined with bifonazole and ketoconazole increase the activity of both of these conventional fungicides. Moreover, the pharmacokinetic profile of the isolated compounds was investigated using computational methods.

KEYWORDS: Antimicrobial activity, antifungal activity, *Quercus ilex*, Fagaceae, oak leaves, proanthocyanidins, Volsurf

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11% are exclusively of plant origin, and a significant number are synthetic drugs obtained from natural precursors.[1] Natural products are still major sources of innovative therapeutic agents for infectious diseases (both bacterial and fungal), cancer, lipid disorders, and immunomodulation.[2] Especially, antibiotics of natural origin provided the means to treat bacterial infections saving millions of individuals.[3] However, during the last 20 years, the problem of antibiotic resistance has emerged. Bacterial and fungal pathogens have evolved numerous defense mechanisms against antimicrobial agents, and nowadays, the need to discover new, more potent antimicrobial agents as accessories or alternatives to antibiotic therapy is stronger. Currently, natural plant compounds are on the focus of some biotechnological companies that are looking for new antimicrobial drugs.[4,5] Plants are a rich source of bioactive compounds with an enormous variety of chemical structures. Such secondary metabolites were developed by the plants in the course of coevolution as a strategy of defense against their predators, and because of similarities in their potential target sites, they may exert beneficial medicinal effects on humans as well.[6,7] In this respect, plant responses against phytophagous insects, fungi, or bacteria could be of use in the search of new potent antimicrobial agents. Although plant pathogenic bacteria and fungi differ from human xenobiotics, they seem to have common mechanisms;[8] therefore, plant defense allochemicals could be used as lead compounds for the development of new stronger and effective antibiotics.

From an ecological point of view, *Quercus* sp. have been one of the classic model systems to study the chemical interactions between plants and herbivorous insects.[9−11] Such interactions are clearly observed in *Quercus ilex* leaves after mite attacks, where abaxial hairs are initially developed and gradually colored with red pigments. Recently, we demonstrated[12] that these morphological alterations are the consequence of qualitative and quantitative alterations in the phenolic content of mite-infected *Q. ilex* abaxial

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trichomes. Mite-attacked (hypertrophic trichomes) leaves contained higher concentrations of procyanidin B3 (**3**), catechin (**5**), and quercetin-3-*O*-glucoside (**6**) than the normal ones.

The aim of this study was the evaluation and comparison of antimicrobial activity of phenolics from *Q. ilex* leaves. For this reason, the substances whose contents changed during mite attacks were chosen for further investigations. However, in the course of extensive phytochemical studies in healthy plant material aimed at creating a secondary metabolite database, a large number of substances were isolated and characterized,[13] belonging to the classes of flavonoids and proanthocyanidins. In addition, high-performance liquid chromatography−ultraviolet−diode array detection−mass spectrometry (HPLC-UV-DAD-MS) analyses of both healthy and mite-infected leaves methanol extracts of *Q. ilex* L. revealed the presence of hydrolyzable tannins, such as vescalagin, castalagin, and acutissimin (A or B), without any significant quantitative difference in both extracts. In this paper, we report on the isolation and structure elucidation of one more A type proanthocyanidin (**1**) from healthy *Q. ilex* leaves, the antimicrobial effects of most isolated phenolics alone and in combination with conventional antibiotics. To complete this study, the most promising compounds were characterized by chemoinformatics tools using the VolSurf approach. From three-dimensional (3D) molecular fields, VolSurf descriptors have been calculated and successively analyzed by principal component analysis. The results highlight the compounds' diversity space and the structural features for possible drug−receptor interactions.

MATERIALS AND METHODS

General Procedures. ^{1}H, ^{13}C, and two-dimensional (2D) NMR spectra were recorded in CD_3OD on two independent Bruker DRX 400 (Bruker, Italia, Bruker, Gmbh, Germany) instruments at 295 K. Chemical shifts are given in parts per million (ppm) and were referenced to the solvent signals at 3.31 and 49.5 ppm for ^{1}H and ^{13}C NMR, respectively. Correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC), and rotating frame Overhauser effect spectroscopy (ROESY) were performed using standard Bruker microprograms. IR spectra were obtained on a Perkin-Elmer PARAGON 500 FT-IR spectrophotometer (Perkin-Elmer, United States). UV spectra were recorded on a Shimadzu UV-160A spectrophotometer (Shimadzu, Japan).[14] ESI mass spectra were measured on a Thermo LTQ Orbitrap (FT-MS^n) (Thermo Fischer Scientific, United States). Optical rotations were measured on a Perkin-Elmer 341 polarimeter (Perkin-Elmer). Circular dichroism (CD) spectra were recorded using a Jasco spectropolarimeter (J-800) operating at room temperature, interfaced with a PC, and analyzed through the standard Jasco software package (Jasco, Japan). Samples were placed in a cuvette of 1 cm path length. Spectra were taken as the average of four accumulations from 200 to 300 nm. The scan rate was 50 nm/min. Spectra were recorded in MeOH with a concentrations of 0.025−0.5 mg/mL. The HPLC analyses were performed using a HP 1100 Liquid Chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a HP 1040 DAD, an automatic injector, an autosampler, and a column oven and managed by a HP 9000 workstation (Agilent Technologies). The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis (see below). Mass spectrometry operating conditions were optimized to achieve maximum sensitivity values: gas temperature, 350 °C, at a flow rate of 10 L/min; nebulizer pressure, 30 psi; quadrupole temperature, 30 °C; and capillary voltage, 3500 V. Full scan spectra from m/z 100 to 1500 in both positive and negative ion modes were obtained (scan time, 1 s). The fragmentor was set at 120 and 180. Separations were performed on a reversed-phase column Purosphers Star RP-18, namely Hibars Prepacked column RT (250 mm × 4.6 mm) with a particle size of 5 mm (Merck, Darmstadt, Germany). The eluents were as follows: water adjusted to pH 3.2 by formic acid (A) and methanol (B). The mobile phase was gradient of A from 95 to 0% (100% B) was run in 25 min, at a flow rate of 0.8 mL/min. The system was operated with oven temperature at 26 °C; the injection volume was 20 μL. Chromatograms were recorded at 254, 280, 330, and 350 nm for both proanthocyanidins and flavonoids. DAD spectra between 200 and 450 nm were stored for all peaks exceeding a threshold of 0.1 mAu. Vacuum-liquid chromatography (VLC) was carried out on silica gel 60H (Merck, Art. 7736), using mixtures of dichloromethane:methanol at increasing polarity 100:0−0:100.[15] Column chromatography (CC) was carried out on Sephadex LH-20 and LH-60 (Pharmacia), using mixtures of methanol:water at different ratios (50:50−100:0).[16] TLC: Merck silica gel 60 F_{254} (Art. 5554). Detection: UV light; spray reagents, anisaldehyde−H_2SO_4.

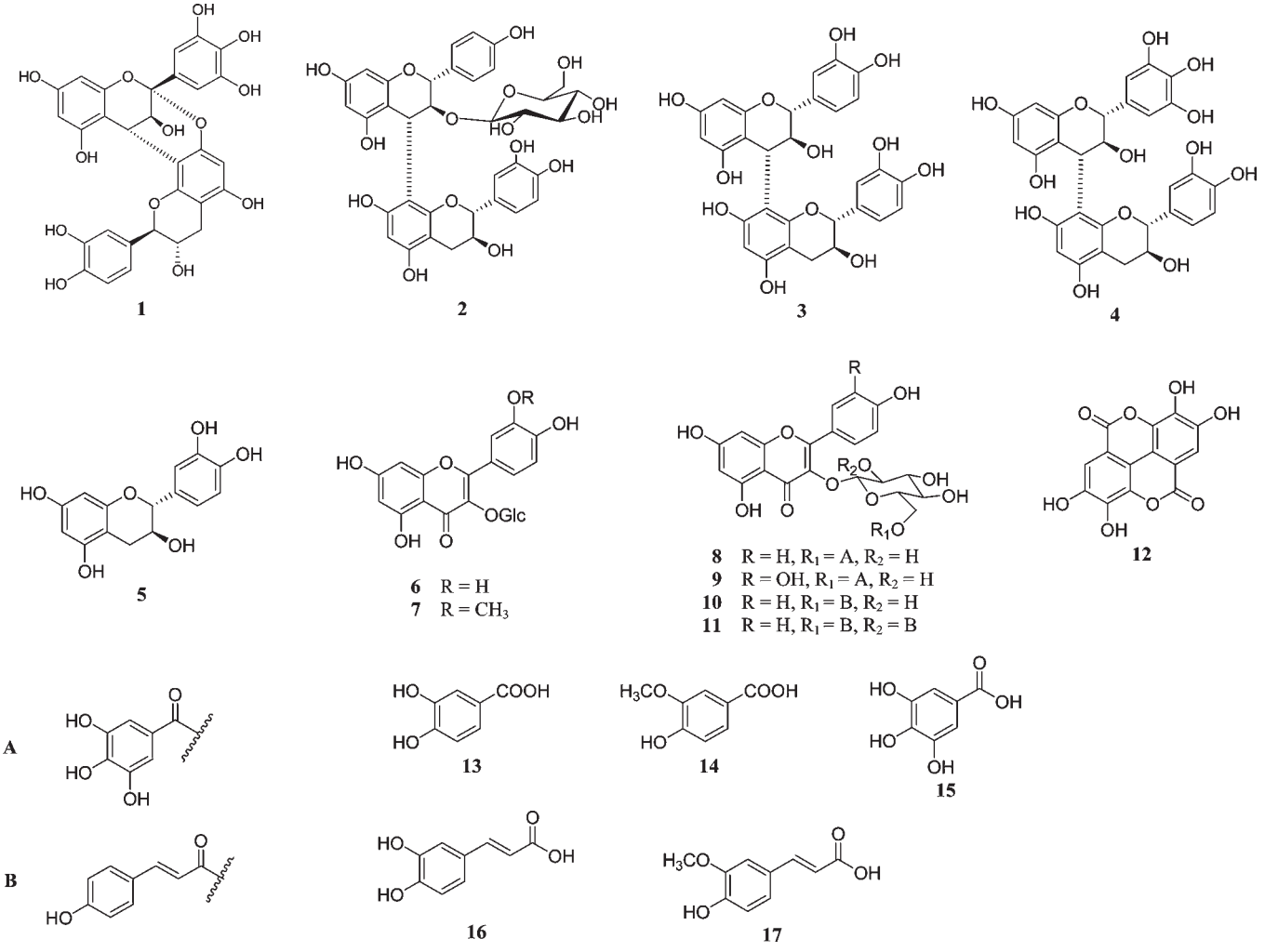
Plant Material. Both healthy and mite-infected leaves of *Q. ilex* L. were collected at Mount Parnes (Attiki, Central Greece) in July 2006. The plant was authenticated by Prof. G. Karabourniotis (Agricultural University of Athens), and a voucher specimen was deposited in the Herbarium of the laboratory of Pharmacognosy and Chemistry of Natural Products under the code Karabourniotis and Skaltsa 01.

Extraction, Isolation, and Identification of the Secondary Metabolites of *Q. ilex* L. Dried healthy leaves of *Q. ilex* (524 g) were successively extracted at room temperature with cyclohexane, Et_2O, MeOH, and MeOH−H_2O 4:1 (3 L of each solvent, three times, 48 h). The dried MeOH extract (57.1 g) was divided in two parts, A (27.3 g) and B (28.9 g). Part A was prefractionated using VLC over silica gel (10 cm × 5 cm) using as eluent CH_2Cl_2−MeOH mixtures of increasing polarity to yield finally eight fractions (QA−QH). Fraction QE (4.48 g; eluted with CH_2Cl_2−MeOH 60:40) was separated in two portions of 2.08 (QE) and 2.4 g (QE′), respectively. Fraction QE further was applied to CC over Sephadex LH-60 using mixtures of MeOH:H_2O (70:30−100% MeOH) and yielded 11 fractions (QEA−QEK). Fraction QEF (211.4 mg; eluted with 70% MeOH) was subjected to CC over Sephadex LH-20 using mixtures of EtOAc−MeOH−H_2O (70:30:10−60:40:10) and afforded procyanidin B_3[17] (**3**) (18.9 mg), prodelphinidin C[18] (**4**) (14.0 mg), and 21.6 mg of impure compound **1**. Purification of the latter fraction by Sephadex LH-20 with 80% MeOH yielded 7.0 mg of pure **1**.

Part B was resuspended in MeOH:H_2O 20:80 and subjected to liquid−liquid partition (three times each solvent) with *n*-hexane and diethylether and then concentrated on a rotary evaporator until methanol evaporation, and the aqueous suspension was extracted repeatedly with EtOAc and *n*-butanol. The diethylether fraction (4.8 g) was subjected to repeated column chromatographies over Sephadex LH-20 with 100% MeOH and afforded after TLC analysis of the eluents five main fractions (QB1−QB5). Fraction QB2 (1.9 g) contained terpenoids and simple phenolics and was subjected to successive Sephadex LH-20 columns using polar (MeOH:H_2O 70:30) and unpolar (EtOAc:MeOH 70:30) solvent systems and yielded after combination three main fractions (QC1−QC3) and 17 mg of impure caffeic acid (**16**) and 23 mg of impure ferulic acid (**17**). Purification over Sephadex LH-20 of those, as well as fraction QC3 (134.9 mg) using different solvent mixtures (50% MeOH−100% MeOH), afforded caffeic acid (5.4 mg) (**16**),[19] ferulic acid (7.2 mg) (**17**),[19] gallic acid (13.9 mg) (**15**),[19] vanillic acid (3.4 mg) (**14**),[19] and protocatechuic acid (6.7 mg) (**13**).[19] Compounds **2** and **5**−**12** have been previously isolated.[13] The known compounds were identified by spectral analysis and direct comparison of their physical properties with those reported previously for these compounds.[13,17−19]

(+)-Epigallocatechin-(2*β*→O→7, 4*β*→8)-(+)-catechin (1). Amorphous light tan powder; $[a]_D^{20}$ +4.07 (c 0.27, MeOH). UV/vis (MeOH) λ_{max} nm: 278. IR (film): ν_{max} cm^{-1}: 3972 (O−H), 2921

Table 1. Structures of Phenolics Isolated from *Q. ilex* L. Leaves



(C−H), 1621 (C═C). For ^{1}H and ^{13}C NMR spectra, see Table 2. HR-ESI-MS *m/z* 591.1135 $[M - H]^-$ (calcd for $C_{30}H_{23}O_{13}$, 591.1138). API-ESI-MS (positive ion mode) *m/z* 593.0 $[M + H]^+$, 615.2 $[M + Na]^+$. CD: ($[\theta]_{204}$ + 2.964, $[\theta]_{212}$ − 4.205, $[\theta]_{229}$ − 1.306).

Biological Assays. Used were the following Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), and *Proteus mirabilis* (human isolate). Used were the following Gram-positive bacteria: *Listeria monocytogenes* (NCTC 7973), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Staphylococcus aureus* (ATCC 6538). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković" (Belgrade, Serbia).

The antibacterial assay was carried out by microdilution method[20−22] to determine the antibacterial activity of compounds tested against the human pathogenic bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. The inocula were prepared daily and stored at +4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtiter plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. Compounds to be investigated were dissolved in broth LB medium (100 μL) with bacterial inoculum (1.0×10^4 CFU per well) to achieve the wanted concentrations (1 mg/mL). The microplates were incubated for 24 h at 48 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial subcultivation of 2 μL into microtiter plates containing 100 μL of broth per well and further incubation for 72 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank and the positive control. Streptomycin and ampicillin were used as positive controls (1 mg/mL DMSO). Two replicates were done for each compound.

For the antifungal bioassays, 14 fungi were used as follows: *Altenaria alternata* Fries, von Keissler (DSM 2006), *Aspergillus flavus* (ATCC 9643), *Aspergillus fumigatus* (plant isolate), *Aspergillus niger* (ATCC 6275), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aureobasidium pullulans* Arnaud de Bary (ATCC 9348), *Cladosporium cladosporioides* Fresenius des Vries (ATCC 13276), *Fulvia fulvum* (TK 5318), *Fusarium sporotrichioides* Sherbakoff (ITM 496), *Fusarium trincintum* Corda, Saccardo (CBS 514478), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112, 5061), and *Trichoderma viride* (IAM). The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology,

Table 2. ^{1}H NMR (CD_3OD, 400 MHz, J in Hz) and ^{13}C NMR (CD_3OD, 100.3 MHz) Spectroscopic Data of Compound 1

ring	position	δ-C	multiplicity	δ-H	HMBC
C	2	100.6	C		
	3	68.1	CH	4.22 d (3.4)	8′, 10
	4	29.4	CH	4.06 d (3.4)	2, 3, 5, 9, 10, 8′, 9′
A	5	156.9	C		
	6	98.4	CH	5.95 d (2.5)	5, 7, 8, 10
	7	158.2	C		
	8	96.8	CH	6.06 d (2.5)	6, 7, 9, 10
	9	154.4	C		
	10	104.3	C		
B	11	131.8	C		
	12	107.7	CH	6.71 s	2, 11, 13, 14, 16
	13	146.2	C		
	14	135.1	C		
	15	146.2	C		
	16	107.7	CH	6.71 s	2, 11, 12, 14, 15
F	2′	84.7	CH	4.73 d (8.2)	3′, 9′, 12′, 16′
	3′	68.3	CH	4.13 m	2′
	4′a	29.2	CH_2	2.94 dd (16.5, 5.8)	2′, 3′, 5′, 9′, 10′
	4′b			2.57 dd (16.5, 8.0)	2′, 3′, 5′, 9′, 10′
D	5′	156.3	C		
	6′	96.7	CH	6.08	5′, 7′, 8′, 10′
	7′	152.5	C		
	8′	107.0	C		
	9′	151.7	C		
	10′	103.3	C		
E	11′	131.0	C		
	12′	116.0	CH	6.91 brs	2′, 13′, 16′
	13′	146.9	C		
	14′	146.6	C		
	15′	116.5	CH	6.81 brs	11′, 12′, 14′
	16′	120.8	CH	6.81 brs	2′, 11′, 14′

Institute for Biological Research "Siniša Stanković" (Belgrade, Serbia). The micromycetes were maintained on malt agar, and the cultures were stored at 4 °C and subcultured once a month.[23] To investigate the antifungal activity of the extracts, a modified microdilution technique was used.[20−22] The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum. MIC determinations were performed by a serial dilution technique using 96-well microtiter plates. The compounds investigated were dissolved in DMSO (1 mg/mL) and added in broth Malt medium with inoculum. The microplates were incubated for 72 h at 28 °C, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MIC. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μL into microtiter plates containing 100 μL of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, and commercial fungicides, bifonazole and ketoconazole, were used as positive controls (1−3000 μL/mL).

Screening Antimicrobial Combinations. The synergism, indifference, and antagonism of the combinations of compound **3** or **4** with different percentages of ketoconazole or bifonazole were initially screened on the same eight fungal strains. The fraction inhibitory concentration index (FICI) was calculated as follows: FIC of compound **3** or **4** (= MIC of compound **3** or **4** combined/MIC of compound **3** or **4**, alone) + FIC (= MIC of compound **3** or **4** combined/MIC of ketoconazole or bifonazole, alone). Synergism was defined as FICI ≤ 0.5; indifferent effect as FICI: 0.5−2 and antagonistic effect as FICI ≥2.[24]

Computational Methods. The molecules were generated using the SYBYL molecular modeling package,[25] and their energies were minimized using the Powell method with a convergent criterion provided by the Tripos force field.[26] Their pharmacokinetic profile was predicted using VolSurf (version 4) (www.moldiscovery.com), a computational procedure that is specifically designed to produce descriptors related to pharmacokinetic properties.[27] The ADME models included in VolSurf predict Caco-2 cell (human intestinal epithelial cell line derived from a colorectal carcinoma) absorption,[27] protein binding,[28] blood−brain barrier (BBB) permeation,[29] drug−water solubility,[30] drug−DMSO solubility, and metabolic stability.[31] We used the probe water (OH_2), hydrophobic (DRY), and H-bonding carbonyl (O) to generate the 3D interaction energies and a Grid space of 0.5 Å. The Gview molecular graphic system (www.moldiscovery.com) was used to visualize the projection of our molecules on the models hERG (human ether-a-go-go related gene) inhibition and volume of distribution.

RESULTS AND DISCUSSION

Compound **1** was obtained as an amorphous light tan powder. The API-ESI-MS spectrum (positive ion mode) of **1** showed pseudomolecular ion peaks at m/z 593.0 $[M + H]^+$, 615.2 $[M + Na]^+$. Its HR-ESI-MS spectrum (negative ion mode) exhibited a molecular peak at m/z 591.1135 $[M - H]^-$ (calcd for $C_{30}H_{23}O_{13}$, 591.1135; Δmmu = 0.3). It yielded a characteristic orange color after spraying with anisaldehyde−sulfuric acid reagent, which is typical of proanthocyanidins. Its UV spectrum (HPLC-DAD) presented a band with maximum at 278 nm. All of the above data suggested that it belonged to the group of catechins/proanthocyanidins. Indeed, a careful look at the MS spectra revealed the presense of a mass fragment at m/z 289.0712, typical of the presense of a catechin moiety, as well as mass fragment at m/z 303.0504 $[M - catechin]^-$. In accordance with the above data, the ^{1}H NMR spectrum showed aromatic signals characteristic of a flavan-3-ol skeleton: All aromatic signals were shifted upfield in the area of δ 6.0−7.0. Most notably, the proton spectrum appeared simplified when compared with those of B type procyanidins (such as procyanidin B3 and prodelphinidin C). This observation along with the presence of an isolated AB system resonating at δ 4.06 and 4.22, with a small coupling constant of 3.4 Hz, suggested an A type of proanthocyanidin. The two doublets, which were assigned to protons H-4 and H-3, respectively, of the C ring, are a diagnostic feature of this type of compounds.[32]

Further 2D NMR experiments (COSY, HSQC, HMBC, and ROESY) enabled the complete identification of the structure. In the aromatic area of the ^{1}H NMR spectrum, two singlets resonating at 6.91 and 6.81 integrating for one and two protons, respectively, were assigned to the ABX system of a catechin moiety. COSY along with HSQC experiments allowed the assignment of the aliphatic protons of ring F and confirmed the presence of a catechin type flavan nucleus, which was unambiguously identified as the lower unit of the dimer. Therefore, the singlet at δ 6.08 was attributed to the ring D of the lower

Table 3. MIC and MBC of Compounds 1−17 (μmol × 10^{-2}/mL)

	MIC MBC																		
bacteria	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	strep	amp
B. cereus	17	14	34	34	35	44	21	17	32	17	14	132	65	119	59	56	52	4.3	24.8
	34	14	68	68	140	88	84	68	64	68	56	132	130	119	236	224	208	8.6	37.2
M. flavus	8.5	14	68	17	140	44	42	68	64	68	28	132	130	119	59	112	208	8.6	24.8
	17	28	68	68	140	88	84	68	64	68	56	132	260	238	236	224	312	17.2	37.2
E. coli	17	14	17	34	35	44	21	17	32	34	28	66	130	119	59	112	104	17.2	37.2
	34	28	68	68	140	88	84	68	64	68	56	132	130	119	236	224	208	34.4	49.2
L. monoc	17	28	68	68	140	88	84	68	64	68	56	33	130	238	118	112	208	25.8	37.2
	34	28	68	102	140	88	84	68	64	68	56	132	260	238	236	224	208	51.6	74.4
P. aerugin	17	14	68	68	140	88	84	68	64	68	28	132	65	119	236	224	208	17.2	74.4
	17	28	68	68	140	88	84	68	64	68	56	132	130	119	236	224	208	34.4	124.0
P. mirabilis	17	3.5	17	17	35	22	21	68	64	17	56	66	130	238	59	112	208	17.2	37.2
	17	7	68	68	140	88	84	68	64	68	56	66	260	238	236	112	208	34.4	49.2
S. aureus	17	28	34	34	35	44	42	34	32	17	28	66	130	119	118	112	104	17.2	24.8
	34	28	68	68	140	88	84	68	64	68	56	132	260	119	236	224	208	34.4	37.2
S. typhimur	17	14	17	17	35	22	21	17	64	68	56	66	65	119	59	56	104	17.2	24.8
	34	14	68	17	140	22	21	68	64	68	56	132	130	119	236	112	208	34.4	49.2

Table 4. MIC and MFC of Compounds 1−17 (μmol × 10^{-2}/mL)

	MIC MFC																		
fungi	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Bif	Keto
Alternaria alternata	8.5	7.0	8.5	8.5	17.5	11.0	10.5	8.5	8.0	6.8	5.6	33.0	65	59	29.5	28.0	26.0	32.0	38.0
	8.5	14.0	8.5	17.0	17.5	11.0	10.5	8.5	8.0	8.5	7.0	66.0	130	119	29.5	28.0	26.0	64.0	76.0
Aspergillus flavus	8.5	7.0	17.0	17.0	35.0	22.0	21.0	17.0	16.0	8.5	14.0	66.0	130	238	59.0	56.0	52.0	48.0	285.0
	17.0	7.0	34.0	34.0	70.0	44.0	42.0	34.0	32.0	8.5	28.0	99.0	260	238	118.0	112.0	104.0	64.0	380.0
A. fumigatus	17.0	14.0	34.0	34.0	35.0	44.0	42.0	34.0	32.0	34.0	28.0	66.0	130	238	118.0	56.0	52.0	48.0	38.0
	34.0	28.0	68.0	68.0	70.0	88.0	84.0	68.0	64.0	68.0	56.0	132.0	260	238	236.0	112.0	104.0	64.0	95.0
A. niger	17.0	14.0	34.0	34.0	70.0	44.0	42.0	34.0	32.0	34.0	28.0	66.0	130	238	118.0	56.0	52.0	48.0	38.0
	34.0	14.0	68.0	68.0	140.0	88.0	84.0	85.0	64.0	68.0	56.0	165.0	260	238	295.0	112.0	104.0	64.0	95.0
A. ochraceus	17.0	14.0	34.0	34.0	70.0	44.0	42.0	34.0	32.0	34.0	28.0	66.0	130	119	118.0	56.0	52.0	48.0	38.0
	34	28.0	51.0	85.0	140	66.0	84.0	85.0	64.0	68.0	56.0	132.0	260	238	295.0	112.0	104.0	64.0	95.0
A. versicolor	17.0	14.0	8.5	8.5	17.5	11.0	10.5	8.5	8.0	6.8	5.6	66.0	65	59	29.5	28.0	26.0	32.0	38.0
	34.0	14.0	17.0	17.0	35.0	11.0	21.0	17.0	8.0	8.5	7.0	99.0	260	238	59.0	56.0	52.0	64.0	95.0
Au. pullulans	8.5	7.0	8.5	8.5	17.5	11.0	10.5	8.5	8.0	6.8	5.6	33.0	65	59	29.5	28.0	26.0	32.0	38.0
	17.0	14.0	8.5	17.0	17.5	11.0	10.5	8.5	8.0	8.5	7.0	66.0	65	59	29.5	28.0	26.0	64.0	76.0
Cl. cladosporioides	8.5	7.0	8.5	8.5	17.5	11.0	10.0	8.5	8.0	5.1	4.2	33.0	65	59	23.6	16.8	20.8	32.0	38.0
	8.5	7.0	8.5	10.2	17.5	11.0	10.5	8.5	8.0	8.5	7.0	66.0	65	59	29.5	28.0	26.0	64.0	95.0
Fulvia fulvum	8.5	7.0	8.5	8.5	17.5	11.0	10.5	8.5	8.0	6.8	5.6	33.0	65	59	29.5	28.0	26.0	32.0	38.0
	8.5	7.0	8.5	17.0	17.5	11.0	10.5	8.5	8.0	8.5	7.0	66.0	130	119	29.5	28.0	26.0	64.0	95.0
Fusarium sporotrichioides	4.2	3.5	8.5	8.5	17.5	8.8	8.4	6.8	6.4	3.4	2.8	33.0	65	59	11.8	16.8	15.6	32.0	38.0
	8.5	7.0	8.5	10.2	17.5	8.8	8.4	8.5	8.0	5.1	4.2	33.0	65	59	17.7	22.4	20.8	64.0	57.0
F. trincintum	4.2	3.5	8.5	8.5	17.5	11.0	8.4	6.8	6.4	5.1	2.8	330	65	59	17.7	16.8	15.0	32.0	38.0
	8.5	7.0	8.5	13.6	17.5	11.0	8.4	8.5	8.0	6.8	5.6	33.0	65	59	23.6	22.4	26.0	64.0	57.0
P. funiculosum	17.0	14.0	17.0	17.0	17.5	44.0	21.0	8.5	32.0	17.0	7.0	33.0	130	119	118.0	28.0	26.0	64.0	38.0
	17.0	28.0	34.0	34.0	35.0	66.0	42.0	17.0	48.0	51.0	14.0	66.0	130	119	236.0	56.0	52.0	80.0	95.0
P. ochlochloron	17.0	14.0	17.0	17.0	17.5	44.0	21.0	17.0	32.0	34.0	28.0	66.0	130	238	59.0	112.0	52.0	48.0	380.0
	34.0	28.0	34.0	34.0	35.0	66.0	42.0	34.0	48.0	51.0	42.0	132.0	260	238	118.0	224.0	104.0	64.0	380.0
Trichoderma viride	8.5	7.0	8.5	8.5	17.5	11.0	10.5	17.0	8.0	8.5	7.0	33.0	65	59	29.5	28.0	26.0	64.0	475.0
	17.0	14.0	17.0	17.0	35.0	22.0	21.0	34.0	16.0	17.0	14.0	66.0	130	238	59.0	56.0	52.0	80.0	570.0

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Table 5. MIC and MFC of Combinations of Compounds 3 or 4 with Different Percentages of Ketoconazole (μg/mL) and Compounds 3, 4, and Ketoconazole (μg/mL)

	MIC MFC								
fungi	**3** + 25% keto	**3** + 50% keto	**3** + 75% keto	**4** + 25% keto	**4** + 50% keto	**4** + 75% keto	**3**	**4**	keto
A. flavus	50	25	25	25	25	200	100	100	1500
	100	50	50	50	50	200	200	200	2000
A. fumigatus	100	200	200	200	100	200	200	200	200
	200	400	400	400	200	200	400	400	500
A. niger	100	200	200	200	200	200	200	200	200
	200	400	400	400	400	400	400	500	500
A. versicolor	25	25	25	25	25	25	50	50	200
	50	50	50	50	50	50	100	50	500
F. fulvum	25	25	100	200	100	25	50	50	200
	50	50	100	200	100	50	50	100	500
P. funiculosum	25	25	25	25	25	25	100	100	200
	50	50	50	50	50	50	200	200	500
P. ochlochloron	25	25	25	25	25	25	100	100	1000
	50	50	50	50	50	50	200	200	1000
T. viride	25	25	25	25	25	25	50	50	2500
	50	50	50	50	50	50	100	100	3000

Table 6. MIC and MFC of Combinations of Compounds 3 or 4 with Different Percentages of Bifonazole (μg/mL) and Compounds 3, 4, and Bifanazole (μg/mL)

	MIC MFC								
fungi	**3** + 25% Bif	**3** + 50% Bif	**3** + 75% Bif	**4** + 25% Bif	**4** + 50% Bif	**4** + 75% Bif	**3**	**4**	Bif
A. flavus	50	100	50	50	200	200	100	100	150
	100	200	400	100	400	400	200	200	200
A. fumigatus	200	100	200	100	25	100	200	200	150
	400	200	400	100	50	400	400	400	200
A. niger	200	100	50	25	25	100	200	200	150
	400	200	400	50	50	400	400	500	200
A. versicolor	200	100	25	50	25	200	50	50	100
	400	200	50	100	50	400	100	50	200
F. fulvum	50	100	25	50	25	25	50	50	100
	200	200	50	100	50	100	50	100	200
P. funiculosum	50	25	25	25	25	200	100	100	200
	200	50	50	50	50	400	200	200	250
P. ochlochloron	200	25	25	100	25	50	100	100	150
	400	50	50	200	50	400	200	200	200
T. viride	100	200	25	25	25	50	50	50	200
	200	400	400	50	50	400	100	100	250

unit, whereas the remaining doublets at δ 5.95 and 6.06 belonged to the upper moiety. The presence of a singlet at δ 6.71 integrating for two protons and corresponding to a carbon at δ 107.7 was typical of a 3′,4′,5′ tri-OH-substituted aromatic ring and indicated the presence of a gallocatechin group. The HMBC spectrum (Figure 1) provided us with substantial data to further correlate gallocatechin and catechin units to the ring systems A,C and D,F, respectively: Common cross-peaks between the protons H-12, 16 (ring B) of the gallocatechin group and the H-4 of the C ring with a quaternary oxygenated carbon at δ 100.6 (C-2) confirmed the presence of an epigallocatechin as the upper part of compound **1**. Therefore, catechin was the lower part of the proanthocyanidin A type skeleton. The 4→8 interflavanoid bond was confirmed by the key correlation [8] between H-4/C-9′ and H-2′/C-9′. Therefore, the linkage between the two units was 2β→O→7, 4β→8, which also explained the upfield shift of C-7 (ring D) when compared with the corresponding one of prodelphinidin C (δ 152.5 vs δ 154.5; data not shown). The ROESY experiment (Figure 1) showed clear interactions between the H-8 and the aromatic protons H-12, H-16 of ring B, H-6′ with

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Table 7. FICI and FIC of Combinations of Different Percentages of Compounds 3 and 4 with Ketoconazole and of Compounds 3 and 4 with Different Percentages of Ketoconazole

	FICI						FIC											
fungi	**3** + keto (25%)	**3** + keto (50%)	**3** + keto (75%)	**4** + keto (25%)	**4** + keto (50%)	**4** + keto (75%)	**3** (25%) + keto	**3** (50%) + keto	**3** (75%) + keto	**4** (25%) + keto	**4** (50%) + keto	**4** (75%) + keto	keto 25% + **3**	keto 50% + **3**	keto 75% + **3**	keto 25% + **4**	keto 50% + **4**	keto 75% + **4**
A. flavus	0.53	0.267	0.267	0.267	0.267	2.133	0.5	0.25	0.25	0.25	0.25	2	0.033	0.017	0.017	0.017	0.017	0.13
A. fumigatus	1	2	2	2	1	2	0.5	1	1	1	0.5	1	0.5	1	1	1	0.5	1
A. niger	1	2	2	2	2	2	0.5	1	1	1	1	1	0.5	1	1	1	1	1
A. versicolor	0.625	0.625	0.625	0.625	0.625	0.625	0.5	0.5	0.5	0.5	0.5	0.5	0.125	0.125	0.125	0.125	0.125	0.125
F. fulvum	0.625	0.625	2.5	5	2.5	0.625	0.5	0.5	2	4	2	0.5	0.125	0.125	0.5	1	0.5	0.125
P. funiculosum	0.375	0.375	0.375	0.375	0.375	0.375	0.25	0.25	0.25	0.25	0.25	0.25	0.125	0.125	0.125	0.125	0.125	0.125
P. ochlochloron	0.275	0.275	0.275	0.275	0.275	0.275	0.25	0.25	0.25	0.25	0.25	0.25	0.025	0.025	0.025	0.025	0.025	0.025
T. viride	0.51	0.51	0.51	0.51	0.51	0.51	0.5	0.5	0.5	0.5	0.5	0.5	0.01	0.01	0.01	0.01	0.01	0.01

Table 8. FICI and FIC of Combinations of Different Percentages of Compounds 3 and 4 with Ketoconazole and of Compounds 3 and 4 with Different Percentages of Bifonazole

	FICI						FIC											
fungi	**3** + Bif (25%)	**3** + Bif (50%)	**3** + Bif (75%)	**4** + Bif (25%)	**4** + Bif (50%)	**4** + Bif (75%)	**3** (25%) + Bif	**3** (50%) + Bif	**3** (75%) + Bif	**4** (25%) + Bif	**4** (50%) + Bif	**4** (75%) + Bif	Bif 25% + **3**	Bif 50% + **3**	Bif 75% + **3**	Bif 25% + **4**	Bif 50% + **4**	Bif 75% + **4**
A. flavus	0.83	1.67	0.83	0.83	3.33	3.33	0.5	1	0.5	0.5	2	2	0.33	0.67	0.33	0.33	1.33	1.33
A. fumigatus	2.33	1.17	1.67	0.17	0.295	1.17	1	0.5	1	0.5	0.125	0.5	1.33	0.67	0.67	0.67	0.17	0.67
A. niger	2.33	1.17	0.455	0.295	0.295	1.17	1	0.5	0.125	0.125	0.125	0.5	1.33	0.67	0.33	0.17	0.17	0.67
A. versicolor	6	3	0.75	1.5	0.75	6	4	2	0.5	1	0.5	4	2	1	0.25	0.5	0.25	2
F. fulvum	1.5	3	0.75	1.5	0.75	0.75	1	2	0.5	1	0.5	0.5	0.5	1	0.25	0.5	0.25	0.25
P. funiculosum	0.75	0.375	0.375	0.5	0.5	3	0.5	0.25	0.25	0.25	0.25	2	0.25	0.125	0.125	0.25	0.25	1
P. ochlochloron	3.33	0.42	0.42	1.17	0.42	0.83	2	0.25	0.25	1	0.25	0.5	1.33	0.17	0.17	0.17	0.17	0.33
T. viride	2.5	5	0.625	0.625	0.625	1.25	2	4	0.5	0.5	0.5	1	0.5	1	0.125	0.125	0.125	0.25

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H-12, H-16 (ring B), and most importantly the cross-peak between H-3 (ring C) and H-6′ (ring D). The latter one is considered to be of diagnostic importance,[33] as it proves further the trans stereochemistry of the 3,4-bond.

The absolute configurations at C-2 and C-4 of **1** were established by CD measurements. The strong negative Cotton effect in the wavelength region between 200 and 230 nm ($[\theta]_{212} - 4.205$, $[\theta]_{204} + 2.964$) indicated the (2α,4α)-configuration.[34−36] As the absolute configuration at position at C-3 was characterized as 3*S* (β- hydroxyl group), based on the NMR data, the absolute configuration at positions 2,3,4 should be 2*R*,3*S*,4*S*; therefore, compound **1** was identified as (+)-epigallocatechin-(2β→O→7, 4β→8)-(+)-catechin.

The isolated compounds (Table 1) were tested against eight human bacterial species, including bacteria food-contaminating species such as *S. typhimurium* and *L. monocytogenes* (Table 3). Compounds **1**−**11** showed higher antibacterial activity (MIC 3.5−140 μmol × 10^{-2}/mL and MBC 7−140 μmol × 10^{-2}/mL) than compounds **12**−**17** (MIC 33−236 μmol × 10^{-2}/mL and MBC 66−312 μmol × 10^{-2}/mL). Instead, methanol extracts of healthy and mite-infected hairs showed low antibacterial activity (data not shown) with MIC of 300−600 μg/mL and MBC of 600 μg/mL (healthy hairs) and MIC of 800−1600 μg/mL and MBC 1600 μg/mL (mite-infected hairs). These results exclude the possibility of synergistic antibacterial effects between the sum of secondary metabolites present in the leaves.

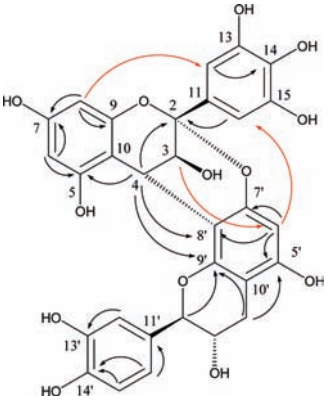

Figure 1. Diagnostic HMBC and ROE correlations for compound **1**.

Among the compounds **1**−**11**, compound **5** exhibits the lowest antibacterial effect. Compounds **1** and **2** possessed the best antibacterial activities. Compounds **1**−**11** showed almost the same bacteriostatic activity but lowest bactericidal activity than streptomycin, while they displayed better antibacterial activity than ampicillin but only against *P. aeruginosa* and *L. monocytogenes*. This is very valuable data since *P. aeruginosa* is known as a very resistant species against all kinds of synthetic and natural antibacterial agents.[37] Concerning the compounds **12**−**17**, compound **17** possessed the lowest antibacterial potential among all of the compounds tested. All of the other compounds in this group showed almost the same antibacterial activity. Compounds **12**−**17** possessed lower antibacterial activity than both antibiotics tested. It can be seen that compounds tested showed different activities on different bacterial species.

The results of the antifungal activity of the isolated compounds are presented in Table 4. The best antifungal potential was obtained by compound **11**, which exhibited the strongest antifungal activity with MIC of 5.6−28.0 μmol × 10^{-2}/mL and MFC of 7.0−56.0 μmol × 10^{-2}/mL. Compounds **1**−**4** and **8**−**10** also possessed strong antifungal activity. Compounds **5** and **7** showed fungistatic activity with slightly lower MIC than previous compounds (10.5−84.0 μmol × 10^{-2}/mL) and fungicidal activity (10.5−140.0 μmol × 10^{-2}/mL). Compounds **12**−**17** exhibited lower antifungal activity (MIC of 26.0−118.0 μmol × 10^{-2}/mL; MFC at 52.0−295.0 μmol × 10^{-2}/mL). Compounds **13** and **15** possessed the lowest antifungal potential. The commercial antifungal agent, ketoconazole, showed fungistatic activity at 38.0−570.0 μmol × 10^{-2}/mL and fungicidal effect at 95.0−570.0 μmol × 10^{-2}/mL, while bifonazole showed MIC at 32.0−64.0 μmol × 10^{-2}/mL and MFC at 64.0−80.0 μmol × 10^{-2}/mL. Compounds **1**−**11** showed higher activity than bifonazole and ketoconazole, except in some cases (compound **5**) against *A. fumigatus* and *A. niger*, where antimycotics reacted slightly better. Almost all compounds tested exhibited good ability to inhibit fungi, even much better than commercial antifungal agents used as reference drugs. Even more, compounds **1**−**11** showed few times higher activity than bifonazole and especially than ketoconazole. These results can be observed against all of the fungal species tested, especially for *Aspergillus versicolor* and *Fulvia fulvum*. The most resistant fungal species were *A. fumigatus* and *A. niger*, where the MIC and MFC of compounds **1**−**11** were

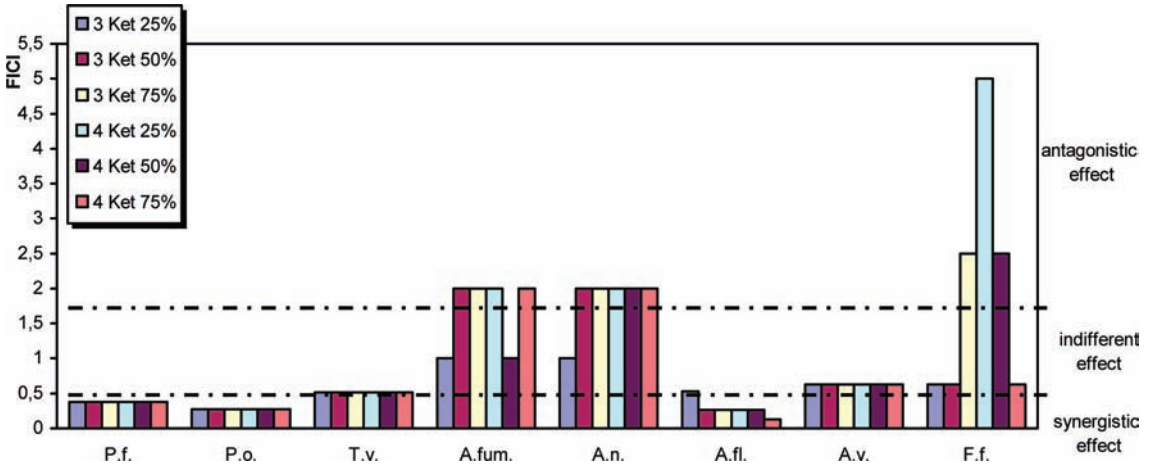

Figure 2. FICI of compounds **3** and **4** and their different percent combinations with ketoconazole.

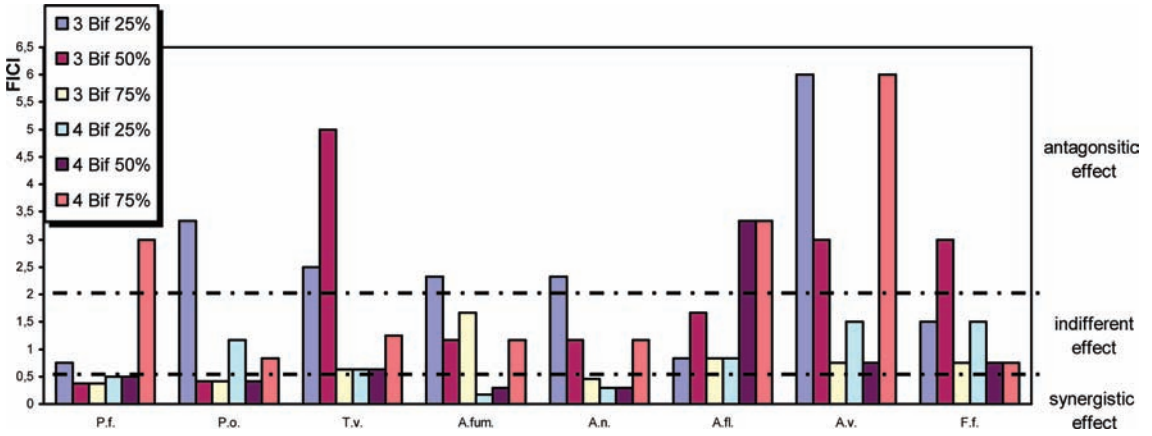

Figure 3. FICI of compounds **3** and **4** and their different percent combinations with bifonazole.

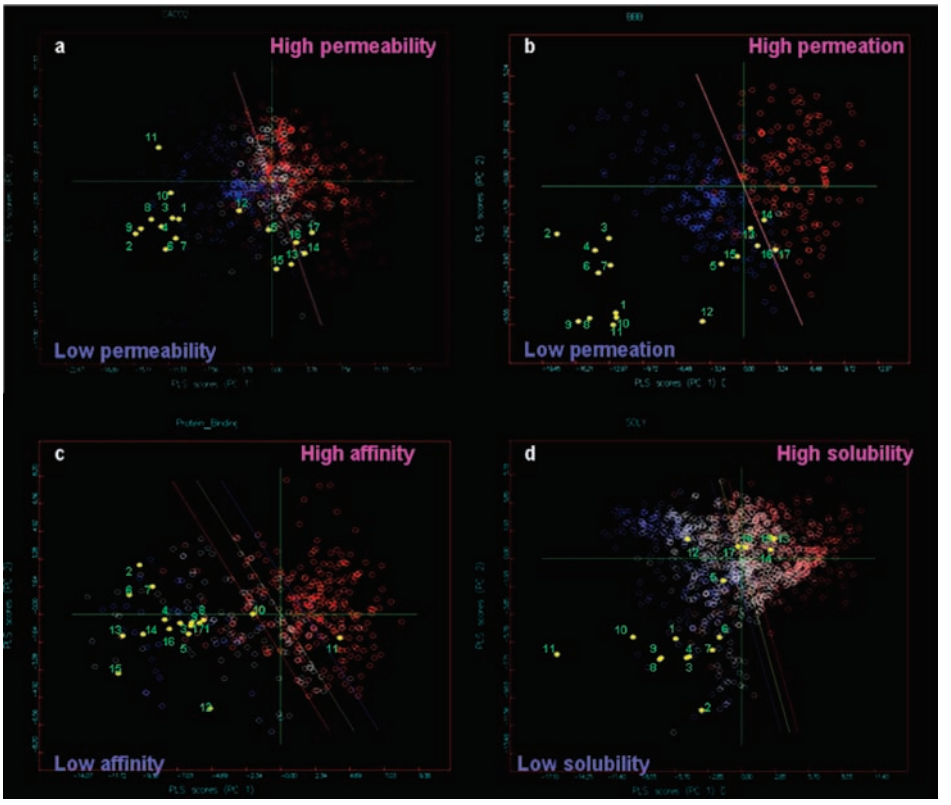

Figure 4. Projection of the studied molecules (yellow-colored circles) on the precalculated models of VolSurf: (a) Caco-2, (b) BBB, (c) protein binding, and (d) solubility.

almost the same or slightly higher than those for bifonazole but lower than values for ketoconazole.

In an attempt to enhance the efficacy of the conventional antifungal drugs, bifonazole and ketoconazole, selected compounds isolated from *Q. ilex* were tested for their possible synergistic effect together with these two well-established antifungal agents.

Among compounds whose concentrations change strongly after mite attack, namely, compounds **3**, **5**, and **6**, only compound **3** proved active against all fungal strains. In addition, compound **4**, even less active, was also tested for potential synergestic effect with commercial antimycotics, as it belongs to the same chemical group of proanthocyanidins.

Therefore, mixtures of compounds **3**/**4** together with bifonazole/ketoconazole were tested against the eight fungal species, which were the most resistant previously. As shown in Table 5, the MIC of ketoconazole (25, 50, and 75%) combined with compound **3** were 25−100, 25−200, and 25−200 μg/mL, respectively. MFCs for this combination were 50−200 (25% ketoconazole) and 50−400 (50 and 75% ketoconazole). The MIC of compound **3** alone was 50−200 μg/mL, while the MFC ranged between 50 and 400 μg/mL. Ketoconazole alone showed

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MIC at 200−2500 μg/mL and MFC at 500−3000 μg/mL. Results clearly indicate a synergy between them. In particular, it can be seen that a higher percentage of ketoconazole in the mixture results in lower inhibition, while a higher percentage of compound **3** enhances the antifungal activity. MIC for all combinations (25, 50, and 75%) of ketoconazole with compound **4** ranged between 25 and 200 μg/mL; and MFCs for these combinations were at 50−400 μg/mL. Compound **4** alone showed MIC at 50−200 μg/mL; MFCs at 50−500 μg/mL. A mixture of compound **4** with ketoconazole 50% showed a higher antifungal activity than those with 25 and 75% ketoconazole. The results of the antifungal activity of combinations of bifonazole with compounds **3** and **4** are presented in Table 6. It can be seen that combination of compound **3** with bifonazole 75% showed better antifungal potential than mixtures containing 25 and 50% of bifonazole, while a combination of compound **4** with 25% bifonazole showed better activity than those containing 50 and 75% bifonazole. From the obtained results, it can be observed that compounds **3** and **4** when combined with bifonazole and ketoconazole increased the activity of both conventional fungicides. For a better understanding of the results, FIC and FICI values are included (Tables 7 and 8 and Figures 2 and 3). In Figure 3, according to FICI, it can be seen that the mixtures tested showed a synergistic effect (≤0.5), an indifferent effect (0.5−2) and an antagonistic effect (≥2). All combinations of ketoconazole with compounds **3** and **4**, separately, showed indifferent effects against *T. viride*, *A. fumigatus*, *A. niger*, *A. versicolor*, and *F. fulvum*; antagonistic effect against *A. fumigatus* (50 and 75% ketoconazole with **3**; 25 and 75% ketoconazole with **4**), against *A. niger* (all mixture except 25% ketoconazole with **3**) and *F. fulvum* (75% ketoconazole with **3**; 25 and 50% ketoconazole with **4**). A synergistic effect was achieved against *P. funiculosum*, *P. ochrochloron*, and *A. flavus* for all of the mixtures of ketoconazole with **3** and **4**, except for 25% ketoconazole with **3**, where an indifferent effect was observed against *A. flavus*. Figure 4 represents the FICI values of bifonazole (25, 50, and 75%) with compounds **3** and **4**, separately. An antagonistic effect was observed for all of the fungi tested. It can be seen that the values raised up to 6, which means that the antagonistic effect of bifonazole and compounds **3** and **4** is very high. A synergistic effect was observed for the mixtures of 50 and 75% bifonazole with **3** and 25 and 50% bifonazole with **4**, against *P. funiculosum*, *P. ochrochloron*, *A. fumigatus*, and *A. niger*. These results indicated that combinations of compounds **3** and **4** with ketoconazole showed a stronger synergistic effect than with bifonazole. In conclusion, we suggest that these combinations may render more effective those conventional fungicides.

Because the isolated compounds have been demonstrated to possess a fungicidal potential higher than the commercial fungicides used as control, we thought that it would be interesting to examine their pharmacokinetic profile using computational methods. The molecules were projected on the precalculated models of VolSurf: Caco-2 cell absorption, plasmatic proteins binding, BBB passage, and thermodynamic solubility. Regarding the Caco-2 cell absorption, the 2D PLS score model offers a discrimination between the permeable and the less permeable compounds. When the spectrum color is active, red points refer to high permeability, and blue points refer to low permeability. From Figure 4a, it is predicted that most of the studied compounds are located in the region of low permeability, which means that they cannot be transported across the intestinal epithelium. The PLS score space of BBB model (Figure 4b) is divided into (left) a region in which BB ranges from negative values until −0.3; this is the region in which compounds show no ability to cross the BBB, (central) a small region from −0.3 to +0.3 (in between red and blue lines) where compounds show moderate permeability, and (right) a region in which BBB ranges from +0.3 value until positive values; this is the region in which compounds show ability to cross the BBB. From the BBB plot, it can be deduced that the projected compounds cannot cross to the BBB. The 2D PLS score model of protein binding (Figure 4c) offers a discrimination between the compounds (colored red) with high protein binding values (between 90 and 100%) and compounds (colored blue) with low protein binding values (from 10 to 50%). The projection of the studied compounds in the protein binding plot suggests a low affinity to the plasma protein, since they are located in the region of compounds with low protein binding values. The PLS plot of aqueous solubility (Figure 4d) shows a differentiation between poorer/low/medium/high/and very high soluble compounds. The projection indicates that the studied compounds are medium-low aqueous soluble. However, it should be noticed that there are several compounds in the models that cover an empty chemical space. For these molecules, the prediction could not be reliable.

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR spectrum of **1** in MeOD, HMBC spectrum of **1**, 3D ChemDraw model for **1**, and CD spectrum of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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