Antimicrobial and Antioxidant Activities of Extracts and Compounds From *Trifolium Baccarinii* With Their Mechanisms of Antibacterial Action

Léonel Feugap
University of Dschang
Irene Kengne
University of Dschang
Jean-De-Dieu Tamokou (jtamokou@yahoo.fr)
University of Dschang
Claudia Ngnokam
University of Dschang
Mahamat Djamalladine
University of Dschang
Laurence Voutquenne-Nazabadioko
Institute of Molecular Chemistry Reims
David Ngnokam
University of Dschang

Research Article

Keywords: Trifolium baccarinii, Fabaceae, flavonoids, saponins, antibacterial, antifungal, antioxidant, mechanisms of action.

Posted Date: December 29th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-130553/v1

Abstract

The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day medicine with many studies showing significant increase in the incidence of bacterial resistance to several antibiotics. The screening of plant extracts and natural compounds for antimicrobial activity has demonstrated that higher plants represent a potential source of new anti-infective agents. The aim of this study was to evaluate the antimicrobial and antioxidant activities of extracts and compounds from the whole plant Trifolium baccarinii Chiov. with their mechanisms of antibacterial action. Biochanin A (1), formononetin (2), luteolin (3), luteolin-4'-O-β-D-glucopyranoside (4), 4,7,2'-trihydroxy-4'-methoxyisoflavanol (5), sissotrin (6), 1-méthyl-β-D-glucopyranoside (7), ononin (8), D-mannitol (9) and 3-O-β-D-glucuronopyranosylsoyasapogenol B (10) were isolated from T. baccarinii. The MeOH, EtOAc and n-BuOH extracts as well as compounds 1–6 from T. baccarinii displayed the most antimicrobial and antioxidant activities. The MeOH extract and compound 5 exhibited antibacterial activity through bacteriolytic effect and reduction of the antioxidant defenses in the bacterial cells. Interestingly, none of the tested samples showed cytotoxic activity against normal cells; highlighting their good selectivity toward pathogenic bacteria and yeasts. Hence, they are promising lead candidates with antibacterial potential against methicillin resistant S. aureus (MRSA).

Introduction

The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day medicine with many studies showing significant increase in the incidence of bacterial resistance to several antibiotics[1]. The expansion of antimicrobial resistance is accelerated by the selective pressure exerted by widespread use and misuse of antimicrobial drugs in both humans and food-producing animals. Antimicrobial resistance is a complex global public health challenge that leads to prolonged illness and increased mortality, increases the costs for the health-care sector, and has an impact on animal health, which probably leads to an effect on food production[2]. As a case in point, over time, the original Gram-positive bacterium *Staphylococcus aureus* developed resistance towards a series of first-line, second-line and even third-line antibiotics[3] to evolve into methicillin-resistant *S. aureus* (MRSA). Now, as MRSA is able to resist the beta-lactam group of antibiotics, the treatment of this highly prevalent pathogen has become an urgent challenge. In organisms, free radicals such as reactive oxygen species (ROS) are formed normally during processes such as cell respiration and have important roles in cell signaling[4]. It is well known that ROS production is rapidly elevated during infection, serving to facilitate pathogen clearance as well as contributing to signaling cascades related to inflammation, cell proliferation, and immune responses[5]. However, high amounts of the free radicals produced during infection can cause oxidative stress; which further complicates the patient's life prognosis.

Due to increased resistance of many microorganisms towards established antimicrobials, much recent attention has been paid to extracts and active principles isolated from plant species used in traditional medicine[6]. The screening of plant extracts and natural compounds for antimicrobial activity has demonstrated that higher plants represent a potential source of new anti-infective agents. Crude extracts from medicinal plants have proved to be clinically effective and relatively less toxic than the existing antibiotics[7]. Phytochemical compounds, particularly flavonoids and other natural compounds play an important role in the defense against free radicals and pathogenic microorganisms[8,9]. Hence, there is a justifiable need to explore for new and more potent antimicrobial/antioxidant compounds of natural origin to combat infectious diseases associated with drug-resistant microorganisms and oxidative stress.

Plants of the genus *Trifolium* are widespread throughout the world and represented in a total of four floristic regions: Neotropic, Paleotropic, Holarctic and Capensis. They are generally small herbaceous plants, some creeping, which can be perennial, annual or biennial. The species of the genus *Trifolium* are characterized by their capacity to fix atmospheric nitrogen through symbiotic bacteria hosted in their roots. The leaves are generally with three leaflets (sometimes four), and are at the origin of the name of the genus. The leaflets are almost always toothed, sometimes smudged at their center. *Trifolium baccarinii* Chiov (Fabaceae) is an annual herb, glabrous or sparingly hairy in upper parts, 7-50 cm tall. The stems are erect, ascending or prostrate and sometimes rooting at nodes in overgrazed areas, grooved and branched. It is a plant of montane grassland at elevations 1,600 m and over in West Cameroon, and widespread in East Cameroon to Ethiopia and East Africa south-wards to Democratic Republic of Congo and Tanzania. In the Cameroonian folk medicine, *T. baccarinii* is used for treatment of various diseases such as dermatosis, pulmonary infections, coughs, fevers and rheumatisms. Some *Trifolium* species are reported to contain isoflavones, flavonoids, pterocarpans, saponins, coumarins, and tyramine[10-13]. The antioxidant, anti-inflammatory and antimicrobial properties of some species of *Trifolium* were determined[12-15]. To date, however, no scientific report could be found in the literature regarding the antioxidant and antimicrobial activities of *T. baccarinii*, although there is an ample ethnobotanical claim for these properties. As a continuing research directed at the biological properties of plants grown in Cameroon, this paper describes the results of the antimicrobial and antioxidant activities of extracts and compounds from *T. baccarinii* with their mechanisms of antibacterial action.

Methods

General experimental procedures

NMR analysis

The ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker Avance III 600 spectrometer equipped with a cryo-platform (^{1}H at 600 MHz and ^{13}C at 150 MHz). 2D NMR experiments were performed using standard Bruker microprograms (Xwin-NMR version 2.1 software). All chemical shifts (δ) are reported in parts per million (ppm) with the solvent signal as reference relative to TMS ($\delta = 0$) as internal standard, while the coupling constants (J) are given in Hertz (Hz). Deuterated solvents, methanol (CD_3OD), dimethyl sulfoxide (DMSO-d_6), and chloroform ($CDCl_3$) were used as solvents for the NMR experiments.

Chromatographic methods

Column chromatography was run on Merck silica gel (VWR, France) 60 (70–230 mesh) and gel permeation on Sephadex LH-20 (VWR, France), while TLC was carried out on silica gel GF254 pre-coated plates and the spots were visualized by an UV lamp multiband UV-254/365 nm (ModelUVGL-58 Upland CA 91786, U.S.A) followed by spraying with 50% H_2SO_4 followed by heating at 100 °C.

Sample collection

The whole plant *Trifolium baccarinii* Chiov was collected on January 2016 in Dschang, western region of Cameroon. The botanical identification was carried out at the National Herbarium of Cameroon after comparison with the samples deposited at the reference number 2976/HNC.

Extraction and Fractionation

The plant material was air-dried at room temperature and ground into fine powder. The dried powder of *T. baccarinii* (4.5 kg) was extracted at room temperature with methanol (3 × 20L, 72h) to yield 281 g of crude methanol extract after evaporation of solvent under reduced pressure. A part of crude extract (271 g) undergoes a differential solubilization with H_2O/EtOAc (300 mL / 500 mL) followed by H_2O/*n*-BuOH (300 mL / 500 mL). After evaporation of each solvent under reduced pressure, we obtained 70 g of EtOAc and 54 g of *n*-BuOH extracts.

Isolation of Compounds

A part of EtOAc extract (65 g) of *T. baccarinii* was subjected to silica gel column chromatography using *n*-hexane-EtOAc (100:0 → 0:100) followed by EtOAc-MeOH (90:10 → 80:20) gradient graduated elution. Sixty-six fractions of 400 mL were collected and combined on the basis of their TLC profiles to give fourteen major fractions: A (1), B (2-6), C (7-10), D (11-14), E (15-20), F (21-25), G (26-31), H (32-34), I (35-40), J (41-46), K (47-52), L (53-58), M (59-60) and N (61-66). Purification of fraction G (8.0 g) on silica gel column chromatography with *n*-hexane-EtOAc (90:10) as eluent yielded compound **1** (27 mg) and **2** (35 mg). Silica gel column chromatography of fraction H (10.0 g) eluted with *n*-hexane-EtOAc (80:20) gave compound **3** (10 mg). Fraction K (5 g) was subjected to silica gel column chromatography eluted with EtOAc to afford seven sub-fractions (K_1-K_7). Compound **4** (10 mg) was obtained from sub-fraction K_3 (250 mg) after Sephadex LH-20 column chromatography using MeOH as eluent. Fraction D (4.2 kg) was subjected to silica gel column chromatography eluted with *n*-hexane-EtOAc (93:7) to yield four sub-fractions (D_1-D_4). Further purification of sub-fraction D_2 on silica gel column chromatography eluted with *n*-hexane-EtOAc (95:5) yield compound **5** (30 mg). The purification of fraction J (3.5 g) on Sephadex LH-20 column chromatography affords two sub-fractions J_1 and J_2. Fraction J_2 (600 mg) was further purified on silica gel column chromatography with *n*-hexane-EtOAc (20:80) to give compound **6** (20 mg).

Like the EtOAc extract, a part of *n*-BuOH extract (50 g) of *T. baccarinii* was subjected to a silica gel column chromatography using the mixture EtOAc-MeOH (100:0 → 40:60) gradient graduated elution. Seventy-six fractions of 400 mL were collected and combined on the basis of their TLC profiles in four major fractions: A (1-7), B (8-23), C (24-41) and D (42-68). Purification of fraction B (5 g) on silica gel column chromatography with EtOAc as eluent mainly leads to compounds **6** (580 mg), **7** (20 mg) and **8** (16 mg). Fraction C (4 g) was purified over a silica gel column chromatography eluted with EtOAc-MeOH-H_2O (90:10:5) to give compound **9** (20 mg). Fraction D (3 g) was subjected to multiple chromatography separation over silica gel using EtOAc-MeOH-H_2O (90:10:5) and (80:20:10) as eluents to give two sub-fractions D_1 and D_2. The latter (D_2, 1 g) was further purified on silica gel column chromatography using EtOAc-MeOH-H_2O (80:20:10) as system and also gave other two sub-fractions $D_{2\text{-}1}$ and $D_{2\text{-}2}$. Purification of sub-fraction $D_{2\text{-}2}$ (200 mg) on Sephadex LH-20 column chromatography using MeOH as eluent yielded compound **10** (20 mg).

Antimicrobial assay

Microorganisms

The antimicrobial activity was performed against five bacterial and two fungal species. The selected microorganisms were the Gram-positive (*Staphylococcus aureus* ATCC25923, methicillin resistant *S. aureus* MRSA03 and methicillin resistant *S. aureus* MRSA04) and Gram-negative (*Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* S2(1)) bacteria and yeast strains of *Candida albicans* ATCC10231 and *Cryptococcus neoformans* H99. These microorganisms were taken from our laboratory collection. The fungal and bacterial strains

were maintained on Sabouraud Dextrose Agar (SDA, Conda, Madrid, Spain) and nutrient agar (NA, Conda) slants respectively.

Determination of minimum inhibitory concentration (MIC) and minimum Microbicidal concentration (MMC)

The antimicrobial activity was investigated by determining the minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFCs). MICs of extracts/compounds were monitored using the broth microdilution method[16.] Test sample was dissolved in dimethylsulfoxide (DMSO, Fisher chemicals) to give a stock solution that was twofold serially diluted in Mueller-Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi to obtain a concentration range of 4096 to 0.25 µg/mL. One hundred microliters of each concentration was introduced into a well (96-wells microplate) containing 90 µL of SDB or MHB and 10 µL of inoculums (at 1 x 10^6 CFU/mL for bacteria and 1 x 10^5 spores/ mL for yeasts) were added to obtain a final concentration range of 2048 to 0.125 µg/mL. Plates were covered and incubated on the shaker at 37 °C, 30 °C and 30 °C during 24 h, 48 h and 72 h for bacteria, *Candida albicans* and *Cryptococcus neoformans*, respectively. MICs were assessed visually after the corresponding incubation period and were taken as the lowest sample concentration at which there was no growth or virtually no growth.

For the minimum microbicidal concentration (MMC) determination, 10 µL aliquots from each well that showed no growth of microorganism were plated on Mueller-Hinton Agar or Sabouraud Dextrose Agar and incubated as described above. The lowest concentration that yielded no growth after the sub-culturing was taken as the MBCs or MFCs. Oxacillin (Sigma-Aldrich, Steinheim, Germany) for bacteria and nystatin (Sigma-Aldrich, Steinheim, Germany) for yeasts were used as positive controls; while broth with 20 µL of DMSO was used as negative control. The assay was carried out in triplicate and repeated three times with similar results.

Antibacterial Mechanism Studies

The mechanisms of antibacterial action were determined by lysis, salt tolerance assays and antioxidant enzyme activities.

Bacteriolytic assay

The bacteriolytic activity of methanol extract and compound **5**, which exhibited the highest antimicrobial activities, was performed against *P. aeruginosa* and *S. aureus* using the time-kill kinetic method as previously described[17] with some modifications. Full growth of bacterium in MHB was diluted 100 times and incubated at 37 °C to produce an OD_{600} of 0.8 as starting inoculum. Sample solutions were added to the starting bacterial suspension to give a final concentration of 2 × MIC and incubated at 37 °C under agitation at 150 rpm. After the incubation period corresponding to 0, 15, 30, 60, 120 and 240 min, 100 µL was removed from each tube and the optical density was recorded at 600 nm using BIOBASE UV-VIS spectrophotometer. Corresponding dilutions of test samples were used as blanks. Oxacillin was used as positive controls and the tubes without extract/compound served as negative controls. All the measurements were done in triplicate and repeated three times with similar results.

Loss of Salt Tolerance in *Staphylococcus aureus.*

The ability of *S. aureus* ATCC25923 and methicillin resistant *S. aureus* MRSA03 cells treated with methanol extract and compound **5** to grow on Mueller Hinton agar (MHA) supplemented with NaCl was investigated. In preliminary experiments, untreated suspensions of *S. aureus* were plated on MHA supplemented with NaCl from 40 to 100 g/L. Plates were incubated and upon incubation, the resulting colonies were counted. Concentrations of NaCl, 50, 60 and

70 g/L, which modestly compromised the colony-forming abilities of untreated microorganisms were selected. For further experimentation steps, suspensions of bacteria were prepared as previously described and treated with MeOH extract or compound **5** at 1/2x MIC, MIC, and 2x MIC. After 1 h incubation, samples were removed, serially diluted, and inoculated onto MHA and MHA-NaCl (50, 60 and 70 g/L). Bacterial culture without sample was used as control for each MHA-NaCl plate. Upon incubation, the numbers of CFU per milliliter on each MHA-NaCl plate were compared to those on the MHA plate, and the result was expressed as a percentage[18].

Antioxidant enzyme activities

For evaluation of catalase and superoxide dismutase (SOD) activities, *S. aureus* ATCC25923 and methicillin resistant *S. aureus* MRSA03 (1.5×10^8 CFU/ml, 500 µl) cultures from the late exponential growth phase were treated with MIC and 1/2xMIC of methanol extract (500 µl), compound **5** (500 µl) and oxacillin (500 µl) solutions and incubated at 37 °C for 24 h. The suspension was centrifuged at 3000 rpm for 5 min to separate the supernatant. Pellet was washed twice with PBS and re-suspended in 500 µl of cell lysate buffer (1 mM EDTA, 10 Mm Tris-HCl, 0.1% Triton-X-100 and 150 mM NaCl)[19] and kept for incubation at 37 °C for 1 h. Contents were then centrifuged at 3000 rpm for 5 min and the supernatant was collected for enzyme activity assays.

Catalase activity was assessed by using kit (Sigma, catalogue no. CAT100) in the cell lysate Briefly, 750 µl of assay buffer (50 mM) was mixed with 25 µl of 50 mM H_2O_2 and 10 µl of cell lysate. The mixture was incubated for 5 min. After that, reaction terminated by the addition of 900 µl of stop solution (15 mM sodium azide) and content was thoroughly mixed. Then, 10 µl of reaction mixture was taken into separate tube and mixed with 1 ml of colour reagent (2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid and 0.25 mM 4-aminoantipyrine) and incubated for 15 min. The absorbance was monitored at 520 nm and the catalase activity was calculated based on the following equation: [Δµ moles (H_2O_2) × d × 100) / V × t], where Δµ moles (H_2O_2) = difference in amount of H_2O_2 added to the reaction mixture between blank and given sample, d = dilution of original sample for catalase reaction, V = sample volume in catalase reaction and t = reaction duration (min).

Superoxide dismutase (SOD) activity was determined using kit (Sigma, Catalogue No. 19160) in the cell lysate. Cell lysate (20 µl) was mixed with working solution of water soluble tetrazolium salt (WST, 200 µl) and enzyme solution (20 µl). The reaction mixture was incubated in dark at 37 °C for 20 min and the absorbance was read at 450 nm on a BioTek Synergy 2 multiplate reader. The SOD activity was calculated based on the following formula: $[(A_{Blank1} - A_{Blank3}) - (A_{Sample} - A_{Blank2}) / (A_{Blank1} - A_{Blank3}) \times 100]$, where Blank 1 contains ultrapure water, WST solution and enzyme solution; Blank 2 contains sample solution, WST solution and dilution buffer whereas Blank 3 contains ultrapure water, WST solution and dilution buffer

Antioxidant assay

Gallic acid equivalent antioxidant capacity (GEAC) assay.

The GEAC test was done as previously described[20] with slight modifications. In a quartz cuvette, to 950 µL acetate buffer (pH = 5.0, 100 mM), the following were added: 20 µL laccase (1 mM stock solution), 20 µL test sample, 10 µL ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (74 mM stock solution). The laccase were purified from *Sclerotinia sclerotiorum* according to the protocol described[21]. The sample concentrations in the assay mixture were 800, 400, 200, 100, 10 µg/mL for the extracts and 200, 100, 50, 25, 12.5 µg/mL for the isolated compounds. The content of the generated $ABTS^{\bullet+}$ radical was measured at 420 nm after 240 s reaction time and was converted to gallic acid equivalents by the use of a calibration curve (Pearson's correlation coefficient: $r = 0.998$) constructed with

0, 4, 10, 14, 28, 56, 84 µM gallic acid standards rather than Trolox. Experiments were done in triplicate and repeated three times with similar results.

Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The free radical scavenging activity of extracts and compounds was evaluated according to described method[22]. The EC_{50} (µg/ml), which is the amount of sample necessary to inhibit by 50% the absorbance of free radical DPPH was calculated[22]. Vitamin C was used as a standard control. All the analyses were carried out in triplicate and repeated three times with similar results.

Cytotoxicity assay

Wistar rats (*Rattus novergicus*) aged 10–12 weeks and weighing 220 to 250 g were randomly selected from our colony. All the procedures and protocols involving animals and their care were conducted in conformity with the institutional guidelines and approved by the Cameroon National Ethical Committee (Reg. No. FWA-IRB00001954) and in compliance with the ARRIVE guidelines. Efforts were also made to minimize animal suffering and to reduce the number of animal used in the experiment. All the rats were sacrificed by intraperitoneal injection of the mixture of ketamine (50 mg/ kg) and xylazine (10 mg /kg) for anaesthesia. Whole blood (10 mL) from albino rats was collected by cardiac puncture into a conical tube containing Ethylene Diamine Tetra Acetic Acid (EDTA) as an anticoagulant. Erythrocytes were harvested by centrifugation at room temperature for 10 min at 1,000 x *g* and were washed three times in PBS buffer[23]. The cytotoxicity was evaluated as previously described[23].

Statistical analysis

Data were analyzed by one-way analysis of variance followed by Waller-Duncan Post Hoc test. The experimental results were expressed as the mean ± Standard Deviation (SD). Differences between groups were considered significant when $p < 0.05$. All analyses were performed using the Statistical Package for Social Sciences (SPSS, version 12.0) software.

Results

Chemical analysis

The purification of the EtOAc and *n*-BuOH extracts from *T. baccarinii* led to the isolation of ten known compounds (Fig. 1). The structures of these compounds were established on the basis of spectroscopic data (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, HMBC, and ROESY). The direct comparison with published information led us to identify those compounds as: Biochanin A **1**[24]; Formononetin **2**[24] ; Luteolin **3**[25] ; Luteolin-4'-O-β-D-glucopyranoside **4**[26] ; 4,7,2'-trihydroxy-4'-methoxyisoflavanol **5**[27] ; Sissotrin **6**[28]; 1-methyl-β-D-glucopyranoside **7**[29] ; Ononin **8**[30] ; D-mannitol **9**[31] and 3-O-β-D-glucurono pyranosylsoyasapogenol B **10**[32].

Antimicrobial activity

The antimicrobial properties of extracts and isolated compounds from *T. baccarinii* were evaluated against both pathogenic bacteria and fungi by determining their minimum inhibitory concentration (MIC) values and the results are depicted in Table 1. The MeOH, EtOAc and *n*-BuOH extracts from *T. baccarinii* were effective in inhibiting the growth of all tested yeasts, Gram-positive and Gram-negative bacteria with MIC values in the range of 32 – 512 µg/mL. The MIC values of MeOH and EtOAc extracts were in the range of 32 - 64 µg/mL, whereas *n*-BuOH extract

was active in the range of 128 - 512 µg/mL towards the tested bacteria and yeasts. This result suggests that the *n*-BuOH extract was lesser active than the MeOH and EtOAc extracts. Indeed, a lowest MIC value indicates a largest antimicrobial agent as less sample is required to inhibit growth of the microorganism. The lowest MIC value of 8 µg/mL was recorded on *C. neoformans* with compound **3** and on *P. aeruginosa, E. coli, S. aureus, C. albicans* and *C. neoformans* with compound **5** whereas the lowest MMC value was obtained on *P. aeruginosa, S. aureus* and *C. neoformans* with compound **5**. However, the highest MIC value of 512 µg/mL was recorded with *n*-BuOH extract against *MRSA04*, while the highest MMC value of 2048 µg/mL was obtained on *MRSA04* with *n*-BuOH extract.

Compounds **1–6** and **8** were active against all the yeasts, Gram-positive and Gram-negative bacteria. By contrast, compound **7** was not active against the tested microorganisms whereas compounds **9** and **10** exhibited low activity and only showed weak inhibition against *P. aeruginosa*, *E. coli*, *S. aureus* and *C. neoformans*. Compound **5** (MIC = 8 – 32 µg/mL) was the most active compound followed in decreasing order by compound **3** (MIC = 8 – 32 µg/mL), compound **4** (MIC = 16 – 64 µg/mL), compound **1** (MIC = 32 – 64 µg/mL), compound **2** (MIC = 64–128 µg/mL), compound **6** (MIC = 32 – 256 µg/mL), compound **8** (MIC = 64 – 256 µg/mL), compound **9** (MIC = ≥ 128 µg/mL), compound **10** (MIC ≥ 256 µg/mL) and compound **7** (MIC >256 µg/mL). As shown in Table 1, oxacillin and nystatin used as standard drugs were more potent than compound **5** against yeasts, Gram-positive and Gram-negative bacteria with the exception against *E. coli* where oxacillin was less active compared with compound **5**. The most susceptible strains towards the tested samples were *P. aeruginosa*, *E. coli*, *S. aureus* and *C. neoformans* whereas the most resistant strains were methicillin resistant *S. aureus* (MRSA03 and MRSA04) and *C. albicans*.

Mechanism of Antibacterial Activity

Bacteriolytic activity

The result on the bacteriolytic activity showed a decrease in the optical density of bacteria suspensions treated with the MeOH extract and compound **5** as a function of time (Fig. 2). Most of the decrease in the optical density was observed during the first periods of incubation (30, 60 and 120 min) followed by a slight decrease in the optical density after 120 min of incubation. After 240 min, the MeOH extract and compound **5** induced a decline in cell turbidity of 97.08 and 99.85% in *P. aeruginosa* suspension and of 99.79 and 99.87% in *S. aureus* ATCC25923 suspension, respectively compared to the 0 time value, indicating the lysis of bacterial cells. Treatment with oxacillin had no effect (decline in cell turbidity of 2% to the 0 time value).

Loss of Salt Tolerance in *Staphylococcus aureus*

The effect of different concentrations of *T. baccarinii* MeOH extract (a) and compound **5** (b) on the reduction of salt tolerance of *S. aureus* is shown in Fig. 3. It can be noted that when the bacteria pretreated with samples were inoculated on culture media supplemented with different concentrations of NaCl, a significant decrease in the number of colony-forming units was observed depending on the pretreatment concentrations of the MeOH extract/compound **5**. Compared to other concentrations, the largest reductions in the number of colonies formed were observed on culture medium supplemented at 70% NaCl with two times the MICs.

Antioxidant enzyme activities

Cell lysates treated with methanol extract, compound **5** and oxacillin showed significant concentration-dependent decreases in catalase and SOD activities compared to those of untreated cell lysates (negative control) (Fig. 4). Also, treatment with MIC of MeOH extract, compound **5** and oxacillin displayed the most significant decreases in catalase and SOS activities when compared to their 1/2xMIC treatment against *Staphylococcus aureus* ATCC25923 and

MRSA03. The effect of compound **5** on the catalase and SOS activities was significantly greater than those of oxacillin and MeOH extract.

Antioxidant activity

The extracts and their isolated compounds were evaluated for their antioxidant activity using DPPH and TEAC methods (Table 2).The DPPH• and ABTS•+ radical scavenging activities were observed in all the extracts. The lowest IC_{50} value reflects the highest DPPH radical scavenging activity whereas the largest gallic acid equivalent antioxidant capacity represents the highest ABTS•+ radical scavenging activity. According to the results obtained, the *n*-BuOH extract was the most potent antioxidant extract followed in decreasing order by the MeOH extract and EtOAc extract. Compounds **7**, **9**, and **10** were found to be inactive in both DPPH and TEAC assays. Compound **4** was the most active antioxidant compound followed in decreasing order by compounds **1**, **2**, **6**, **3**, **5** and **8**.

Cytotoxic activity

The cytotoxic activity of extracts and isolated compounds from *T. baccarinii* against red blood cells (RBCs) was investigated using Triton X-100 as a positive control. The positive control showed about 100% lysis, whereas the phosphate buffer saline (PBS) showed no lysis of RBCs. Interestingly, none of the tested extracts and compounds showed cytotoxic activity against RBCs at concentrations up to 2048 µg/mL for the extracts and 256 µg/mL for the isolated compounds (results not shown).

Discussion

The antimicrobial activity of the MeOH extract was comparable with that of the EtOAc extract but higher than that of the *n*-BuOH extract, indicating that fractionation decreased the antimicrobial activity of the *n*-BuOH extract and did not affect that of the EtOAc extract. The findings of the present study revealed that the MeOH, *n*-BuOH and EtOAc extracts from *T. baccarinii* showed different degrees of antimicrobial activities against bacterial and fungal strains. Differences observed in the antimicrobial activities of extracts can be linked to the differences in their chemical composition whereas variations in the susceptibility of tested microorganisms can be explained by the genetic differences between the strains. The result of the present study reveals the potential of *T. baccarinii* as a source of antibacterial and antifungal drugs and provides scientific evidence for its use in folk medicine for the treatment of various infectious diseases. To our knowledge, no previous publications have reported the antibacterial and antifungal activities of *T. baccarinii*. So, this plant can be used as a novel therapeutic agent to prevent the progress of various infectious diseases particularly those caused by the tested microorganisms. In addition, this is the first study using the spectroscopic methods for identification of chemical constituents from *T. baccarinii* in which known compounds have been identified for the first time in this plant. According to the antimicrobial cutoff points defined in the literature for plant extract[33], the MeOH and EtOAc extracts of *T. baccarinii* were highly active (MIC < 100 µg/mL) against all the tested microorganisms whereas the *n*-BuOH extract was significantly active (100 ≤ MIC ≤ 512 µg/mL) against the tested microorganisms.

The microbicidal properties of extracts and isolated compounds against susceptible strains were analysed by the minimum microbicidal concentration (MMC) assay. Indeed, an antimicrobial agent is considered microbicidal if the MMC is not more than fourfold higher than the MIC, i.e. MMC/MIC ≤ 4[22]. The MeOH, EtOAc and *n*-BuOH extracts as well as compounds **1–6** and **8** were shown to be microbicidal (MMC/MIC ≤ 2) against the susceptible microorganisms with exception of the *n*-BuOH extract against *E. coli* and methicillin resistant *S. aureus* (MRSA04); compounds **6** and **8**, against methicillin resistant *S. aureus* (MRSA03 and MRSA04), *C. albicans* and *C. neoformans*,

with the MMC values being eightfold higher than the MIC indicating bacteriostatic character. These results suggest that the bacteriostatic/fungistatic and bactericidal/fungicidal activities of the *n*-BuOH extract and compounds **6** and **8** are dependent on the microbial strain. This behaviour is different to standard antibiotics, oxacillin and nystatin, which displayed microbicidal activities (MMC/MIC ≤ 4) against all the tested microorganisms.

Considering the antimicrobial cutoff points of pure compounds defined in a previous report[33], the antimicrobial activities of test compounds could be considered as significant/moderate (for compounds **3** and **5**); moderate (for compounds **1** and **4**); moderate/low (for compounds **2**, **6** and **8**); low/not active (for compounds **9** and **10**); not active for compound **7** against specific microorganism.

All of the compounds that were found to be active in the present study are members of saponins and flavonoid groups. Although saponin and flavonoid compounds have been reported to possess antibacterial and antifungal activities[9,21], no study has reported the activity of compounds **1-6, 8** and **10** on the types of pathogenic microbial strains used in the present study. The mechanism of action of saponin (**10**) is not fully understood, but it may involve membrane disruption by lipophilic compounds[34]. The mechanism of action of flavonoids (**1**–**6**, **8**-**9**) is still to be studied; nevertheless, their activity may be due to the disruption of microbial membranes and their ability to complex bacterial cell walls, extracellular and soluble proteins[35]. With regard to the structure–activity relationship analysis, it is suggested that the number and position of hydroxyl, sugar and methoxy groups in flavanone skeleton of compounds **1–4, 6, 8** and **10** are responsible for different degrees of antibacterial, antifungal and antioxidant activities observed.

The results of the present study demonstrated that the *n*-BuOH extract was the most potent antioxidant extract among the extracts whereas compounds **1–4** and **6** were the most active antioxidant compounds. This finding suggests that the *n*-BuOH extract and compounds **1– 4** and **6** are the best candidates to combat diseases associated with oxidative stress. These results are consistent since reactive oxygen species (ROS) generated from activated neutrophils and macrophages have been reported to play an important role in the pathogenesis of various diseases, including neurodegenerative disorders, cancer and atherosclerosis[36]. This is very promising in terms of discovering antioxidants from plants. Previous studies recorded that phenolic compounds including flavonoids and their glycosides are associated with strong antioxidant activity and they possess healthy benefits[22,37]. Finally, the phytoconstituents and antioxidant / antimicrobial properties of extracts and isolated compounds from *T. baccarinii* are now well established in this study. These results clearly justify the uses of *T. baccarinii* in the treatment of various infectious diseases caused by the tested microorganisms and other ailments associated with oxidative stress.

The result on the bacteriolytic activity showed a decrease in the optical density of bacteria suspensions treated with the MeOH extract and compound **5** as a function of time; indicating the lysis of bacterial cells. Interestingly, none of the tested extracts and compounds showed cytotoxic activity against normal cells. These findings highlight the selective toxicity of the tested samples towards the studied microorganisms. Previous studies demonstrated that some antimicrobial agents cause gross membrane damage[9,38]; and this has been reported previously for flavonoid glycosides from *G. grandulosum*[9] and for essential oils from rosewood, oregano, and thyme[38]. The effect of MeOH extract and compound **5** to lyse *P. aeruginosa* and *S. aureus* cells suggests that their primary mechanism of action is gross cell wall damage.

Sublethal injury of microbial cell membranes may alter their permeability and affect the membrane's ability to exclude toxic materials or to osmoregulate the cell adequately[39]. Consequently, the loss of tolerance to salts or other

potentially toxic compounds may be exploited to reveal membrane damage[40] in sublethally injured bacteria. Treatment of *S. aureus* with different concentrations of MeOH extract/compound **5** significantly reduced the number of colony-forming units on media containing NaCl. This effect was most marked on culture medium supplemented at 70% NaCl with two times the MICs. These results correlate well with the bacteriolysis results since, in each case, treatment with MeOH extract / compound **5** at two times the MICs induced the loss of salt tolerance and bacteriolytic effect.

Treatment with MeOH extract/compound **5** caused reduction of superoxide dismutase (SOD) level in *S. aureus* strains. Indeed, SOD, which catalyzes the dismutation of superoxide into hydrogen peroxide is the first line defence in bacterial cells against reactive oxygen species[41]. *S. aureus* was facilitated by two major SODs such as SOD-A and SOD-M, the former is mainly involved in endogenous stress while the latter is induced in exogenous stress[41]. Suppression of SOD activity results in decreased conversion of O_2- to H_2O_2 and consequently resulting in increased O_2-levels and leads to oxidative stress mediated toxicity in *S. aureus* cells. Early reports demonstrated decreases in SOD activity in *E. coli* and *S. aureus* upon treatment with methanolic extract of *A. paniculata*, 2-phenylethynylbutyltellurium (PEBT) and catechin[42-44].

Treatment with MeOH extract/compound **5** also induced depletion of catalase activity in *S. aureus* cell suspension. Only one type of catalase (Kat-A) was found in *S. aureus* which is involved in the detoxification of H_2O_2 by converting it into H_2O and O_2 with the help of heme co-factor[45]. Hence, decrease of catalase level caused by MeOH extract/compound **5** might result in increased H_2O_2 level and leads to oxidative stress mediated toxicity in *S. aureus* cell suspension. Similar to our findings, *Leonurus cardiaca* extract and other phyto-compounds like silibin, allylpyrocatechol and catechin also reduced the catalase activity and caused toxicity in *S. aureus*[44, 46-48]. Altogether results suggest that the MeOH extract and compound **5** are promising lead candidates with antibacterial potential against MRSA. Thus, these samples exhibited antibacterial activity through bacteriolytic effect and reduction of the antioxidant defenses in the bacterial cells.

Given the results obtained in the present study, we can conclude that the purification of the EtOAc and *n*-BuOH extracts from *T. baccarinii* led to the isolation and characterization of ten known compounds namely: biochanin A (**1**), formononetin (**2**), luteolin (**3**), luteolin-4'-O-β-D-glucopyranoside (**4**), 4,7,2'-trihydroxy-4'-methoxyisoflavanol (**5**), sissotrin (**6**), 1-methyl-β-D-glucopyranoside (**7**), ononin (**8**), D-mannitol (**9**) and 3-O-β-D-glucuronopyranosylsoyasapogenol B (**10**). The MeOH, EtOAc and *n*-BuOH extracts as well as compounds **1–6** from *T. baccarinii* displayed the most antimicrobial and antioxidant activities. The MeOH extract and compound **5** exhibited antibacterial activity through bacteriolytic effects and reduction of the antioxidant defenses in the bacterial cells. To the best of our knowledge, this is the first report on the mechanisms of antibacterial action of MeOH extract and compound **5** from *T. baccarinii* against pathogenic strains. Interestingly, none of the tested extracts/compounds showed cytotoxic activity against normal cells; highlighting their good selectivity toward pathogenic bacteria and yeasts. The MeOH extract and compound **5** are promising lead candidates with antibacterial potential against MRSA. Hence, they can be utilized to fight against infectious diseases caused by the tested microorganisms and to combat diseases that induce oxidative stress.

Declarations

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgements

Not applicable.

Authors' contributions

LDTF, ICK, CDJN and MDD contributed to the data collection and analysis. JDT designated the study, did the biological assays and helped in manuscript writing and editing. JDT, LVN and DN supervised and revised the manuscript critically for important intellectual content. All authors read and agreed on the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

References

1. Kumarasamy, Y., Cox, P. J., Jaspars, M, Nahar, L. & Sarker, S. D. Screening seeds of Scottish plants for antibacterial activity. *J Ethnopharmacol.***83**, 73-77 (2002).
2. World Health Organization (WHO). Antimicrobial Resistance Global Report on Surveillance. WHO Press, Geneva (2014).
3. Theuretzbacher, U. Resistance drives antibacterial drug development. *Curr Opin Pharmacol.* **11**, 433–438 (2011).
4. Crimi, E*. et al.* The role of oxidative stress in adult critical care. *Free Radic Biol Med.***40**, 398-406 (2006).
5. Sareila, O., Kelkka, T., Pizzolla, A., Hultqvist, M. & Holmdahl, R. NOX2 complex derived ROS as immune regulators. *Antioxid Redox Signal.* doi: 10.1089/ars. (2010).
6. Essawi, T. & Srour, M. Screening of some Palestinian medicinal plants for antibacterial activity. *J Ethnopharmacol.***70**, 343-349 (2000).
7. Awaad, A. S., El-Meligy, R. M., Qenawy, SA, Atta, A. H., & Soliman, G. A. Anti-inflammatory, antinociceptive and antipyretic effects of some desert plants. *J Saudi Chem Soc.***15**, 367–373 (2011).
8. Joubouhi, C., Tamokou, J. D., Ngnokam, D., Voutquenne-Nazabadioko, L. & Kuiate, J. RIridoids from *Canthium subcordatum* iso-butanol fraction with potent biological activities. *BMC Compl Altern Med.***17**, 17 (2017).
9. Tagousop, N. C., Tamokou, J. D., Ekom-Endeguele, S., Ngnokam, D. & Voutquenne-Nazabadioko, L. Antimicrobial activities of flavonoid glycosides from *Graptophyllum grandulosum* and their mechanism of antibacterial action. *BMC Compl Altern Med.***18**, 252 (2018).
10. Booth, L. N. *et al.* The chemical and biological profile of a red clover (*Trifolium pratense*) Phase II Clinical Extract. J Altern Complement Med. **12**, 133–139 (2006).
11. Sharaf, M. Chemical constituents from the seeds of *Trifolium alexandrinum. Nat Prod Res.***22**, 1620-1623 (2008).
12. Flythe, M. & Kagan, I. Antimicrobial Effect of Red Clover (*Trifolium pratense*) Phenolic extract on the ruminal hyper ammonia-producing bacterium, *Clostridium sticklandii*. *Curr Microbiol.***61**, 125-131 (2010).

13. Vlaisavljević, S., Kaurinović, B., Popović, M. & Vasiljević, S. Profile of phenolic compounds in *Trifolium pretense* extracts at different growth stages and their biological activities. *Int J Food Properties***20**, 3090–101 (2017).
14. Nielsen, T. S., Höjer, A., Gustavsson, A. M, Hansen-Møller, J & Purup, S. J. Proliferative effect of whey from cows'milk varying in phyto-oestrogens in human breast and prostate cancer cells. *J Dairy Sci.***79**, 143-149 (2012).
15. Liu, R. & Xu, B. Inhibitory effects of phenolics and saponins from commonly consumed food legumes in China against digestive enzymes pancreatic lipase and α-glycosidase. *Int J Food Properties* **18**, 2246–255 (2015).
16. Pagning, N. A. L. *et al.* New triterpene and new flavone glucoside from *Rhynchospora corymbosa* (Cyperaceae) with their antimicrobial, tyrosinase and butyrylcholinesterase inhibitory activities. *Phytochem Lett.***16**, 121-128 (2016).
17. Ooi, N. *et al.* XF-73 a novel antistaphylococcal membrane active agent with rapid bactericidal activity. *J Antimicrob Chemother.***64**, 735-740 (2009).
18. Patra, J. K. & Baek, K. H. Antibacterial activity and action mechanism of the essential oil from *Enteromorpha linza* against food borne pathogenic bacteria. *Molecules***21**, 388 (2016).
19. Martins, D. *et al.* Superoxide dismutase activity confers (p)ppGppmediated antibiotic tolerance to stationary-phase *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA***115**, 9797–802 (2018).
20. Rice-Evans, C. & Miller, N. J. Total antioxidant status in plasma and body fluids. *Methods Enzymol.***234**, 279-293 (1994).
21. Mot, A. C. e*t al.* "Yellow" laccase with "blue" spectroscopic features, from *Sclerotinia sclerotiorum*. *Process Biochemistry***47**, 968–975 (2012).
22. Djouossi, M. G. *et al.* Antimicrobial and antioxidant flavonoids from the leaves of *Oncoba spinosa* (Salicaceae). *BMC Compl Altern Med.***15**, 134 (2015).
23. Situ, H. & Bobek, L. A. *In vitro* assessment of antifungal therapeutic potential of salivary histatin-5 two variants of histatin-5 and Salivary Mucin (MUC7) domain 1. *Antimicrob Agents Chemother.* **44**, 1485-493 (2000).
24. Solange, A. D. S. & Màrio, G. D. C. Unambiguous ^{1}H- and ^{13}C-NMR Assignment of isoflavone from *Virola caducifolia*. *J Braz Chem Soc.***6**, 349-352 (1995).
25. Muraleedharan, G. N., Gene, R. S. & Jose, O. S. Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover (*Trifolium repens*) roots. *Appl Environ Microbiol.***57**, 434-439 (1990).
26. Ufuk, Ö. *et al.* Relationship between chemical structure and antioxidant activity of Luteolin and its glycosides isolated from *Thymus sipyleus sub sp. Sipyleus var. Sipyleus*. *Record Nat Prod.***5**:12-21 (2010).
27. Se, Y. K. *et al.* Flavonoid Glycosides as Acetylcholinesterase Inhibitors from the whole plants of *Persicaria thunbergii*. *Nat Prod Sci.***20**, 191-195 (2014).
28. Bojase, G., Cornelius, C. W. W. & Runner, R. T. M. Two new isoflavanoids from *Bolusanthus speciosus*. *Bull Chem Soc Ethiopia***15**:131-136 (2001).
29. Vitor, R. F. *et al.* Flavonoids of an extract of *Pterospartum tridentatum* showing endothelial protection against oxidative injury. *J Ethnopharmacol.***93**, 363–370 (2004)
30. Aubert, S. *et al.* Methyl-*β*-D-glucopyranoside in higher plants: accumulation and intracellular localization in *Geummontanum* leaves and in model systems studied by ^{13}C nuclear magnetic resonance. *J Exp Bot.***55**, 2179–2189 (2004).
31. Francisco, E. A. R. *et al.* Diterpene and other constituents from *Stemodia maritima* (Scrophulariaceae). *J Brazilian Chem Soc.***21**, 1581-1586 (2010).

32. Matulova, M., Bilik, V. & Alfoldi, J. Reactions of saccharides catalyzed by molybdateions XXXVIII. NMR spectra of alditols in molybdate complexes. *Chem Papers* **43**, 403-414 (1998).
33. Tamokou, J. D., Mbaveng, T. A. & Kuete, V. Antimicrobial activities of African medicinal spices and vegetables. In: In: *Medicinal spices and vegetables from Africa: therapeutic potential against metabolic, inflammatory, infectious and systemic diseases.* 1st edn, Chap 8. Academic Press, Cambridge, Elsevier, Pp 207–237 (2017).
34. Termentzi, A., Fokialakis, N. & Skaltsounis, A. L. Natural resins and bioactive natural products thereof as potential antimicrobial agents. *Curr Pharm Des.***17**, 1267-1290 (2011).
35. Cowan, M. M. Plant product as antimicrobial agents. *Clin Microb Rev.***12**, 564-582 (1999).
36. Winrow, V. R., Winyard, P. G., Morris, C. J. & Blake, D. R. Free radicals in inflammation: second messengers and mediators of tissue destruction. *British Med Bull.***49**, 506-522 (1993).
37. Nzogong, T. R. *et al.* Antimicrobial and antioxidant activities of triterpenoid and phenolic derivatives from two Cameroonian Melastomataceae plants: *Dissotis senegambiensis* and *Amphiblemma monticola*. *BMC Compl Altern Med.***18**, 159 (2018).
38. Horne, D., Holm, M., Oberg, C., Chao, S. & Young, D. G. Antimicrobial effects of essential oils on *Streptococcus pneumoniae*. *J Essential Oil Res.***13**, 387-392 (2001).
39. Gilbert, P. The revival of microorganisms sublethally injured by chemical inhibitors, p. 175–197. In M. H. E. Andrews and A. D. Russell (ed.), The revival of injured microbes. Academic Press, London, United Kingdom (1984).
40. Iandolo, J. J. & Ordal, Z. J. Repair of thermal injury of *Staphylococcus aureus. J Bacteriol.***91**, 134–142 (1996).
41. Beavers, W. N. & Skaar, E. P. Neutrophil-generated oxidative stress and protein damage in *Staphylococcus aureus*. *Pathog Dis.***74**, DOI: https://doi.org/10.1093/femspd/ftw060 (2016).
42. Hussain, R. M., Razak, Z. N. R. A., Saad, W. M. M. & Mustakim, M. Mechanism of antagonistic effects of *Andrographis paniculata* methanolic extract against *Staphylococcus aureus*. *Asian Pac J Trop Med.***10**, 685-695 (2017).
43. Pinheiro, F. C. *et al.* Antimicrobial effect of 2-phenylethynyl-butyltellurium in *Escherichia coli* and its association with oxidative stress. *J Microbiol Biotechnol.***28**, 1209–1216 (2018).
44. Sinsinwar, S. & Vadivel, V. Catechin isolated from cashew nut shell exhibits antibacterial activity against clinical isolates of MRSA through ROS-mediated oxidative stress. *Appl Microbiol Biotechnol.* org/10.1007/s00253-020-10853-z. (2020).
45. Staerck, C. *et al.* Microbial antioxidant defense enzymes. Microb Pathog. **110**, 56-65 (2017).
46. Hussain, R. M., Abdullah, N. F. & Amom, Z. Killing of *Staphylococcus aureus* by allylprocatechol is potentiated by induction of intracellular oxidative stress and inhibition of catalase activity. *J Integr Med.***14**, DOI: https://doi.org/10.1016/S2095-4964(16)60279-0 (2016).
47. Micota, B. *et al*. Is it true that plant-derived polyphenols are always beneficial for the human? *In vitro* study on *Leonurus cardiac* extract properties in the context of the pathogenesis of *Staphylococcus aureusJ Med Microbiol*.**65**, 1171–1181 (2016).
48. Cai, J. Y. et al. Concentration-dependent dual effects of silibinin on kanamycin-induced cells death in *Staphylococcus aureus*. *Biomed Pharmacother.***102**, 782–791 (2018).

Tables

Table 1. Antimicrobial activity (MIC and MMC in µg/mL) of extracts and isolated compounds from *T. baccarinii* as well as reference antimicrobial drugs. /: not determined; MIC: Minimum Inhibitory Concentration; MMC Minimum Microbicidal Concentration; *: nystatin for yeasts and oxacillin for bacteria.

Extracts/ Compounds	Inhibition parameters	*P. aeruginosa*	*E. coli*	*S. aureus*	*MRSA03*	*MRSA04*	*C. albicans*	*C. neoformans*
MeOH extract	MIC	32	64	32	32	64	64	32
	MMC	64	128	32	64	64	128	64
	MMC/MIC	2	2	1	2	1	2	2
EtOAc extract	MIC	64	64	32	32	32	64	32
	MMC	128	128	64	64	64	256	128
	MMC/MIC	2	2	2	2	2	4	4
n-BuOH extract	MIC	256	128	256	128	512	128	128
	MMC	512	1024	512	512	2048	256	128
	MMC/MIC	2	8	2	4	4	2	1
1	MIC	32	32	32	64	64	32	32
	MMC	64	64	32	128	128	64	32
	MMC/MIC	2	2	1	2	2	2	1
2	MIC	128	64	64	128	128	64	64
	MMC	128	128	128	256	256	128	64
	MMC/MIC	1	2	2	2	2	2	1
3	MIC	32	32	32	16	32	16	8
	MMC	64	32	64	16	64	32	16
	MMC/MIC	2	1	2	1	2	2	2
4	MIC	64	32	32	64	64	16	16
	MMC	128	32	64	128	128	16	16
	MMC/MIC	2	1	2	2	2	1	1
5	MIC	8	8	8	16	16	8	8
	MMC	8	16	8	32	16	16	8
	MMC/MIC	1	2	1	2	1	2	1
6	MIC	128	64	64	256	256	64	32
	MMC	256	128	128	≥256	≥256	≥256	256
	MMC/MIC	2	2	2	/	/	/	8
7	MIC	≥256	≥256	≥256	≥256	≥256	≥256	≥256

	MMC	▯256	▯256	▯256	▯256	▯256	▯256	▯256
	MMC/MIC	/	/	/	/	/	/	/
8	MIC	256	128	64	256	256	128	64
	MMC	256	256	256	▯256	▯256	▯256	▯256
	MMC/MIC	1	2	4	/	/	/	/
9	MIC	256	128	128	▯256	▯256	▯256	256
	MMC	▯256	▯256	▯256	▯256	▯256	▯256	▯256
	MMC/MIC	/	/	/	/	/	/	/
10	MIC	256	256	256	▯256	▯256	▯256	256
	MMC	▯256	▯256	▯256	▯256	▯256	▯256	▯256
	MMC/MIC	/	/	/	/	/	/	/
Ref*	MIC	2	16	1	4	8	1	2
	MMC	2	32	1	8	8	1	2
	MMC/MIC	1	2	1	2	1	1	1

Table 2. Antioxidant activities of extracts and some of isolated compounds from *T. baccarinii* EC_{50}: Equivalent concentrations of test samples scavenging 50% of DPPH radical; ND: Not determined. Data represent the mean ± SD of three independent experiments carried out in triplicate. In the same column, values affected by different superscript letters (a-j) are significantly different according to one way ANOVA and Waller Duncan test; $p < 0.05$.

Extracts/compounds	DPPH free radical scavenging activity (EC_{50})	Gallic acid equivalent antioxidant capacity (GEAC)
MeOH extract	98.26 ± 0.74[a]	35.59 ± 0.63[a]
EtOAc extract	105.13 ± 0.86[b]	27.43 ± 1.01[b]
n-BuOH extract	81.74 ± 0.98[c]	46.14 ± 1.26[c]
1	4.87 ± 0.43[d]	101.77 ± 1.24[d]
2	6.39 ± 0.62[e]	77.91 ± 0.59[e]
3	8.02 ± 1.16[e]	68.30 ± 0.71[f]
4	3.71± 0.99[d]	118.71 ± 0.19[g]
5	11.58 ± 0.73[f]	61.05 ± 0.90[h]
6	7.13 ± 0.97[e]	72.96 ± 1.54[i]
8	38.96 ± 1.54[g]	57.43 ± 0.97[j]
Vitamin C	1.81 ± 0.19[h]	ND

Figures

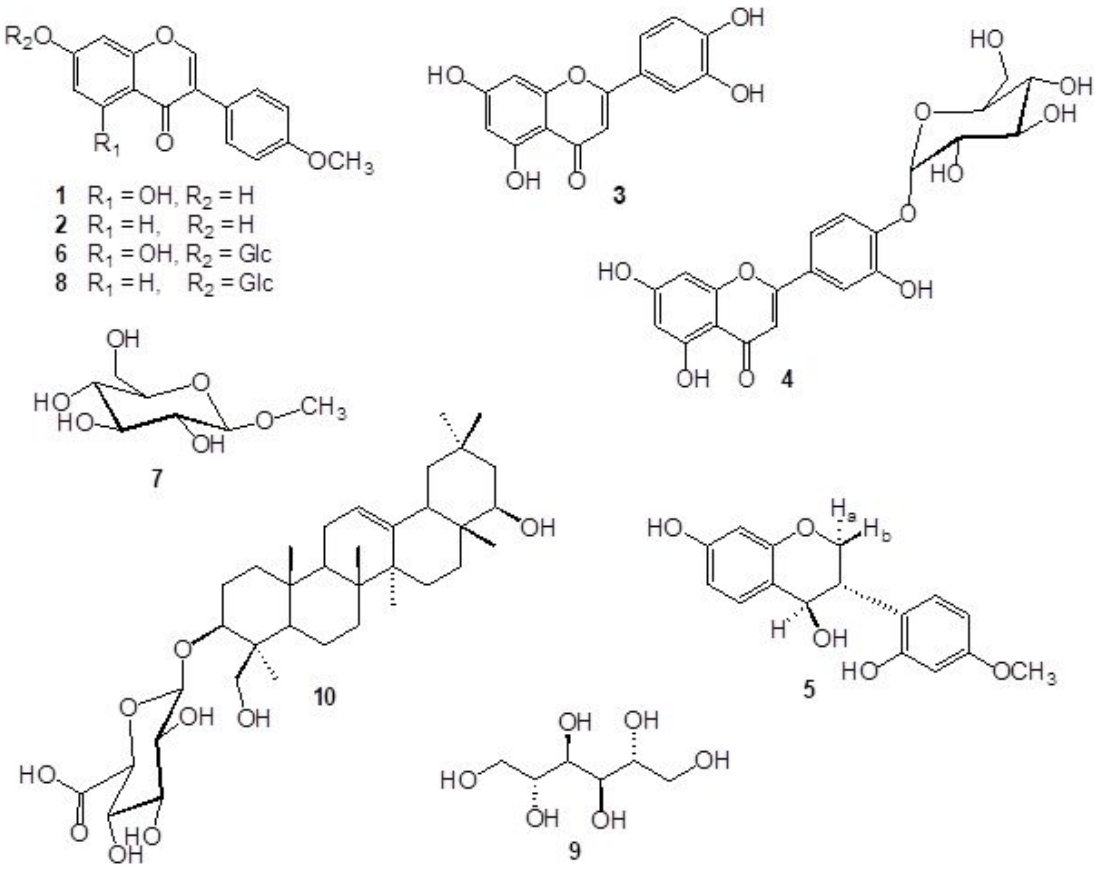

Figure 1

Chemical structures of compounds isolated from T. baccarinii (1–10). 1: Biochanin A; 2: Formo-nonetin; 3: Luteolin; 4: Luteolin-4'-O-β-D-glucopyranoside; 5: 4,7,2'-trihydroxy-4'-methoxyisoflavanol; 6: Sis-sotrin; 7: 1-méthyl-β-D-glucopyranoside; 8: Ononin; 9: D-mannitol; 10: 3-O-β-D-glucuronopyranosylsoyasapogenol B;

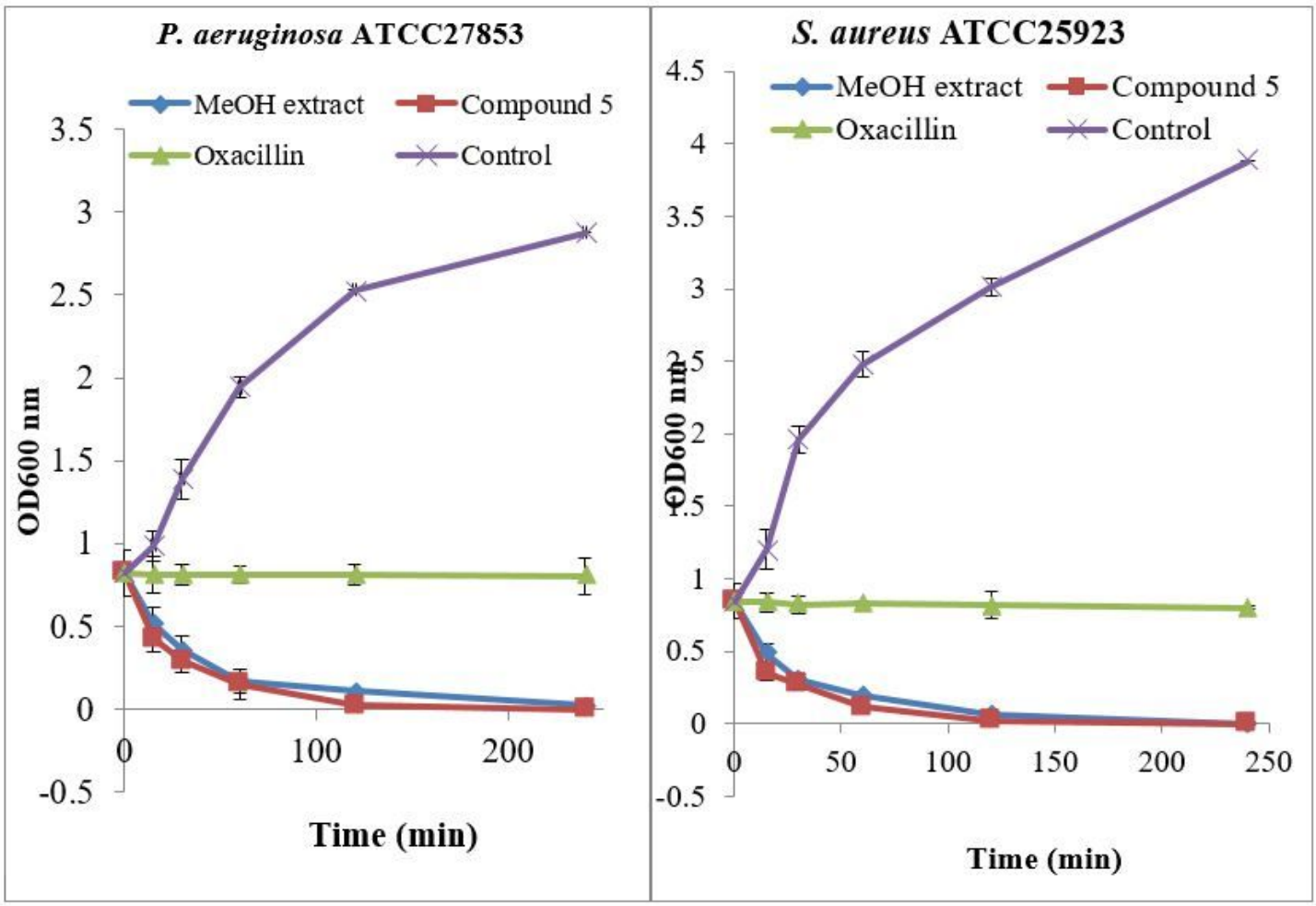

Figure 2

Bacteriolytic effect of MeOH extract and compounds 5 against P. aeruginosa and S. aureus. Results represent the mean ± standard deviation of the triplicate OD at each incubation time.

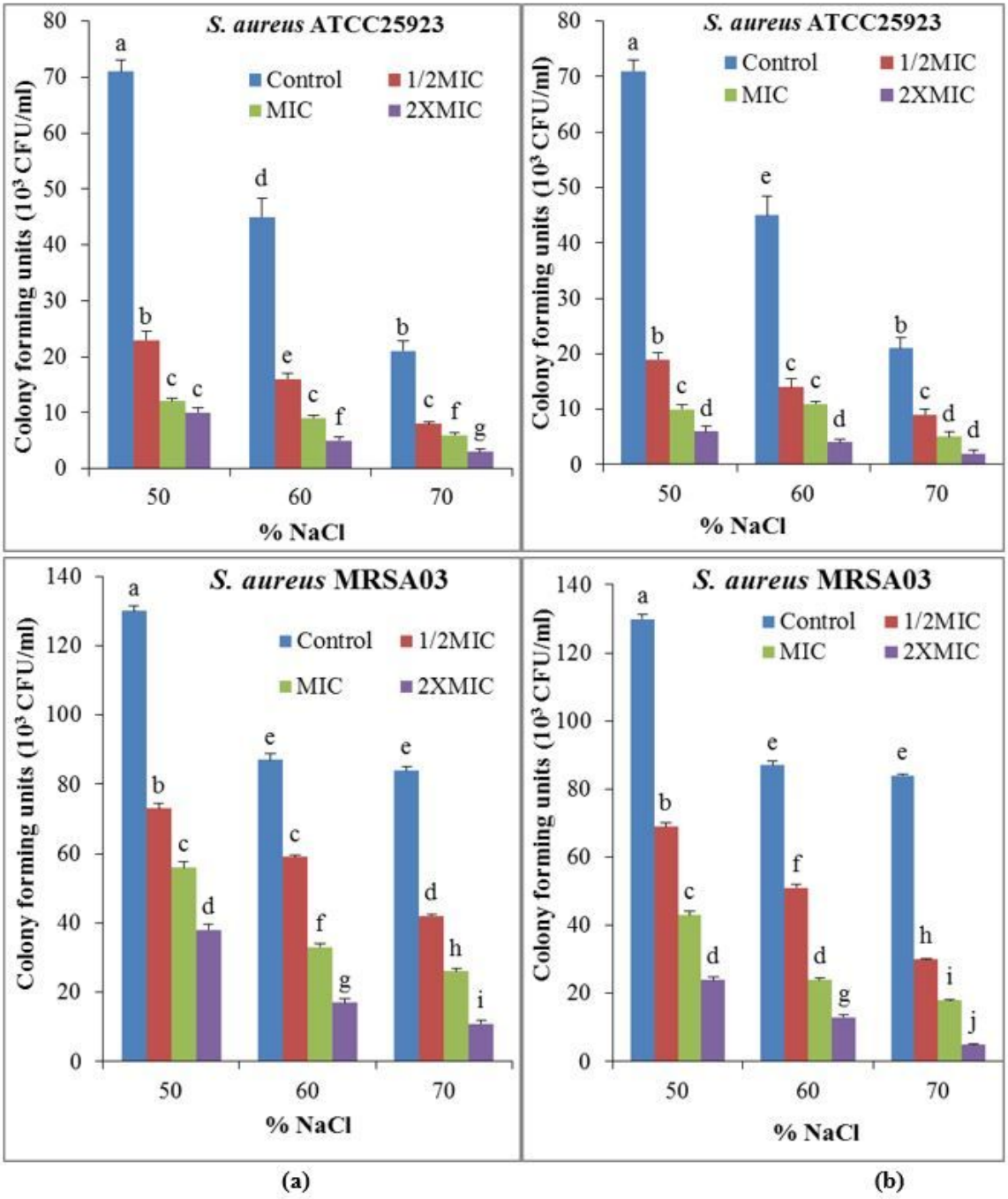

Figure 3

Effect of T. baccarinii methanol extract (a) and compound 5 (b) on the reduction of salt toler-ance of Staphylococcus aureus. MIC: minimum inhibitory concentration; Bars represent the mean ± standard deviation of the triplicate CFU. Values with different letters are significantly different at $p < 0.05$ ac-cording to Waller Duncan test at $p < 0.05$.

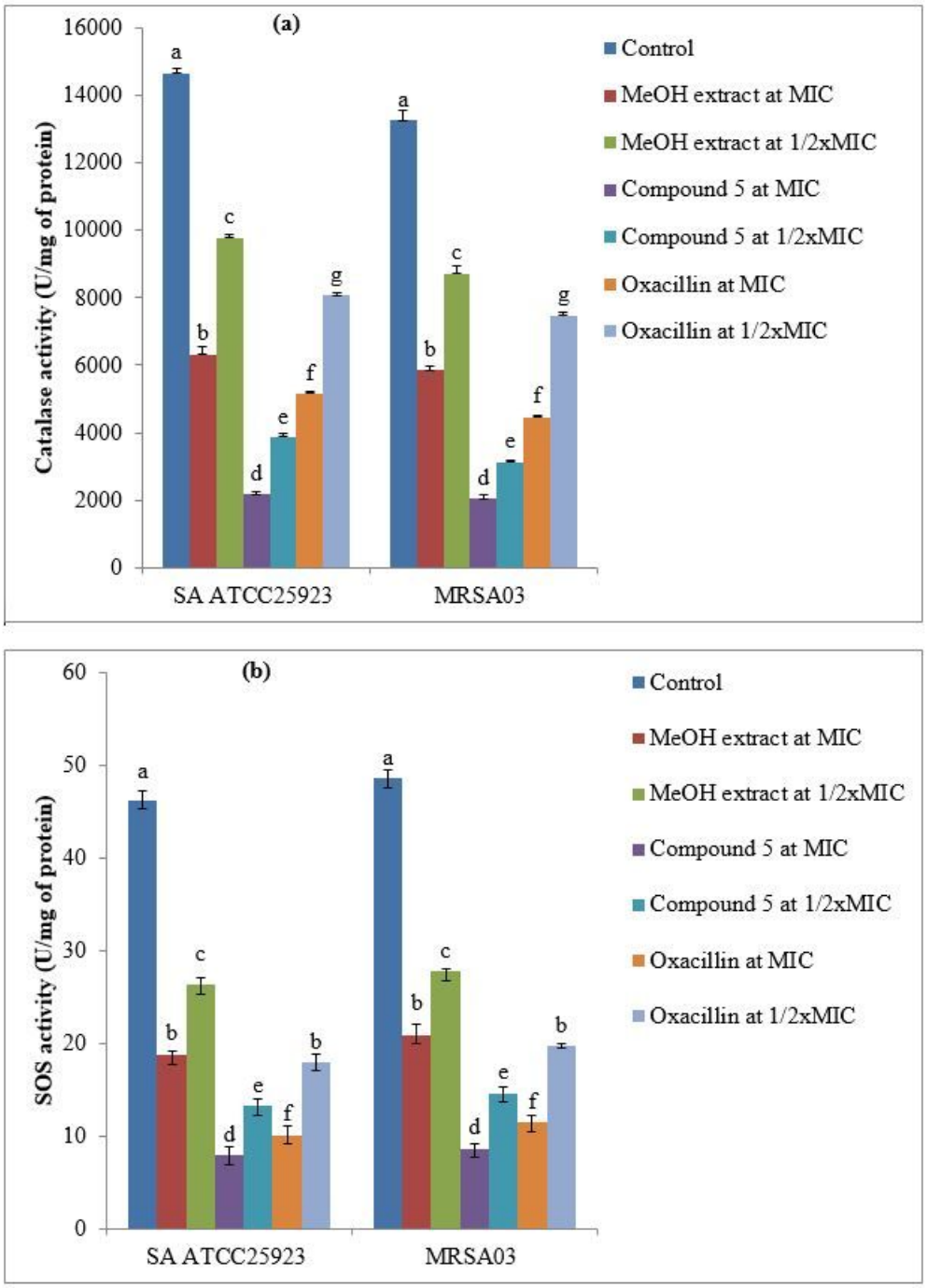

Figure 4

Antioxidant catalase (a) and superoxide dismutase (b) activities in Staphylococcus aureus ATCC25923 and MRSA03 treated with MeOH extract and compound 5. SA ATCC25923: Staphylococcus aureus ATCC25923; MRSA03: methicillin resistant S. aureus 03; Bars represent the mean ± SD of three independent experiments carried out in triplicate. For the same microorganism and enzyme, values affected by different superscript letters (a-g) are significantly different according to one way ANOVA and Waller Duncan test; $p < 0.05$.