Antimicrobial activity of the methanolic extracts and compounds from *Treculia obovoidea* (Moraceae)

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Abstract

The crude extract from *Treculia obovoidea* was subjected to purification by repeated chromatography. Eight compounds were isolated from *Treculia obovoidea* and identified as Psoralen (**1**), Bergapten (**2**), 7-methoxycoumarin (**3**), 7-hydroxycoumarin (**4**), 4,2′,4′-trihydroxychalcone (**5**), 4,2′,4′-trihydroxy-3-prenylchalcone (**6**), 3-hydroxy-4-methoxybenzoic acid (**7**) and *O*-[3-(2,2-dimethyl-3-oxo-2*H*-furan-5-yl) butyl] bergaptol (**8**). These compounds together with the extract were tested for their antimicrobial activity against Gram-positive bacteria (six species), Gram-negative bacteria (12 species) and three *Candida* species using micro-dilution methods for the determination of the minimal inhibition concentration (MIC) and the minimal microbicidal concentration (MMC). The MIC values obtained with the crude extracts varied from 78.12 to 156.25 μg/ml against 17 (80.95%) of the 21 tested microorganisms. All the isolated compounds showed selective activity. The antimicrobial activity of this plant as well as that of compounds **6** and **8** is being reported for the first time. The obtained results provide promising baseline information for the potential use of these crude extract as well as some of the isolated compounds in the treatment of bacterial and fungal infections.

Keywords: *Treculia obovoidea*; Moraceae; Compounds; Antimicrobial activity

1. Introduction

The biologically active compounds research from plant has always been of great interest for scientists, looking for new sources of useful drugs against infectious diseases. Many medicines have come from botanical sources. Approximately 25% of the active substance prescriptions in the United States come from plant material (Céspedes et al., 2006). It is estimated that an amount of 20,000 species from several families is useful for these purposes (Penso, 1982). Our herbal medicine researches include plants of the Moraceae family. *Treculia obovoidea* N.E. Brown (Moraceae), one of the tree plants of the genus *Treculia* is traditionally used to treat skin diseases, dental allergy, amoebic dysentery and AIDS (Berg et al., 1985; Bokesch et al., 2004). It is distributed in the humid regions of Africa, from Nigeria to Congo. The aim of this investigation was to evaluate the antibacterial and anticandidal activities of the crude extracts and compounds isolated from *Treculia obovoidea*.

2. Methodology

2.1. Plant material

The twigs of *Treculia obovoidea* N.E. Brown in August 2004 in Kumba, South-West Province of Cameroon were collected.

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The botanical identification of the plants was done at the Cameroon National Herbarium, where the voucher specimen was conserved under the reference number 44055/HNC.

2.2. Purification and general procedures

The air-dried and powdered twigs of *Treculia obovoidea* (1.5 kg) were macerated in methanol (5 l) at room temperature for 24 h. The filtrate was concentrated under vacuum to give a dark green crude extract (TOT) (80 g).

A part of TOT (65 g) was subjected to vacuum liquid chromatography (VLC) on silica gel (60, 200 g) and eluted with petrol 40–60/ethyl acetate mixtures, EtOAc and EtOAc–MeOH mixtures to give 35 fractions of 250 ml each. The fractions were monitored by TLC and similar fractions were combined. Fractions 1–15 (9 g), eluted with petrol–EtOAc (9:1) examined on TLC with the same solvent system contained mainly mixtures of hydrocarbons and phytosterols. Recrystallisation of these fractions yielded Psoralen (**1**) (22 mg; M_W: 185) (Kuster et al., 1994); Bergapten (**2**) (18 mg; M_W: 216, m.p.: 188) (Kuster et al., 1994). Fractions 16–21 (15 g), eluted with petrol–EtOAc 20% and 30%, respectively, afforded compound **2** (10 mg), 7-methoxycoumarin (**3**) (16 mg; M_W: 176; m.p.: 117–118) (Oliva et al., 2003) and 7-hydroxycoumarin (**4**) (17 mg; M_W: 162) (Oliva et al., 2003). The combined fractions 22–35 (8 g) eluted with 70% petrol–EtOAc were passed through a Sephadex LH-20 column and eluted with $CHCl_3$:MeOH (2:1) mixture. The post-chlorophyll fractions were combined and subjected successively to silica gel CC and preparative TLC to give: 4,2′,4′-hydroxychalcone (**5**) (14 mg; M_W: 256) (Jez and Noel, 2002); 4,2′,4′-trihydroxy-3-prenylchalcone (**6**) (13 mg; M_W: 324) (Asada and Yoshikawa, 1998); isovanilic acid or 3-hydroxy-4-methoxybenzoïc acid (7) (13 mg; M_W: 168; m.p.: 247–250) (Cartwright and Smith, 1967; Hwu et al., 1997) and *O*-[3,(2,2-dimethyl-3-oxo-2*H*-furan-5-yl)butyl]bergaptol (**8**) (15 mg; M_W: 368) (Kuster et al., 1994).

Aluminium sheet pre-coated with silica gel 60 $F_{254\,nm}$ (Merck) was used for thin layer chromatography and the isolated spots were visualized using both ultra-violet light (254 and 366 nm) and 50% H_2SO_4 spray reagent. The chemical structure of each of the isolated compound was determined on the basis of spectral data produced by one and two-dimensional nuclear magnetic resonance (NMR), recorded on Brüker DRX-400 instrument. This spectrometer was equipped with 5 mm, ^1H and ^{13}C NMR probes operating at 400 and 100 MHz, with tretramethylsilane as internal standard. Mass spectra were recorded on an API QSTAR pulsar mass spectrometer. The structures of the compounds were confirmed by comparing with reference data from available literature.

2.3. Microbial strains

Twenty one species of microorganisms namely *Bacillus cereus* LMP0404G, *Bacillus megaterium* LMP0204G, *Bacillus stearothermophilus* LMP0104G, *Bacillus subtilis* LMP0304G, *Staphylococcus aureus* LMP0206U, *Streptococcus faecalis* LMP0207U (Gram-positive bacteria), *Escherichia coli* LMP0101U, *Shigella dysenteriae* LMP0208U, *Proteus vulgaris* LMP0103U, *Proteus mirabilis* LMP0504G, *Shigella flexneri* LMP0313U, *Klebsiella pneumoniae* LMP0210U, *Pseudomonas aeruginosa* LMP0102U, *Salmonella typhi* LMP0209U, *Morganella morganii* LMP0904G, *Enterobacter aerogens* LMP 1004G, *Citrobacter freundii* LMP0904G, *Enterobacter cloacae* LMP1104G (Gram-negative bacteria), *Candida albicans* LMP0204U, *Candida gabrata* LMP0413U and *Candida krusei* LMP0311U (yeasts) were used in this study. Three *Bacillus* species were provided by 'Institut Appert de Paris', while *Bacillus cereus* was provided by the A.F.R.C Reading Laboratory of Great Britain. Other strains were clinical isolates from 'Centre Pasteur du Cameroon', Yaoundé. The microbial isolates were maintained on agar slant at 4 °C in the Laboratory of Applied Microbiology and Molecular Pharmacology (LMP) (Faculty of Science, University of Yaoundé I) where the antimicrobial tests were performed. The strains were sub-cultured on a fresh appropriate agar Plate 24 h prior to any antimicrobial test.

2.4. Antimicrobial assays

2.4.1. Culture media

Nutrient Agar (NA) containing Bromocresol purple was used for the activation of *Bacillus* species while NA was used for other bacteria. Sabouraud Glucose Agar was used for the activation of the fungi. Nutrient broth containing 0.05% phenol red and supplemented with 10% glucose (NBGP) was used for MIC and MMC determinations. The Mueller Hinton Agar (MHA) was also used for the determination of the MMC.

2.4.2. Chemicals

Nystatin (Maneesh Pharmaceutic Pvt. Ltd., Govandi, Mumbai 400043, India) and gentamycin (Jinling Pharmaceutic (Group) Corp., Zhejang Tieng Feng Pharmaceutic Factory, No. 11 Chezhan Road, Huzhou City, Zhejang, China) were used as reference antibiotics (RA) against yeasts and bacteria, respectively.

2.4.3. MIC and MMC determinations

The MICs of test samples and RA were determined as follows: the test sample was first of all dissolved in dimethylsulfoxide (DMSO). The solution obtained was added to NBGP to a final concentration of 156.25 μg/ml for each sample and RA. This was serially diluted two-fold to obtain concentration ranges of 0.31–156.25 μg/ml. The RA solutions (in DMSO) were also prepared following the same concentration ranges as above. One hundred microliters of each concentration was added in a well (96-wells microplate) containing 95 μl of NBGP and 5 μl of inoculum (standardised at 1.5×10^6 CFU/ml by adjusting the optical density to 0.1 at 600 nm SHIMADZU UV-120-01 spectrophotometer) (Tereschuk et al., 1997). The final concentration of DMSO and Tween in the well was less than 1% (preliminary analyses with 1% (v/v) DMSO/NBGP and 10% (v/v) Tween 20/NBGP affected neither the growth of the test organisms nor the change of color due to this growth). The negative control well consisted of 195 μl of NBGP and 5 μl of the standard inoculum

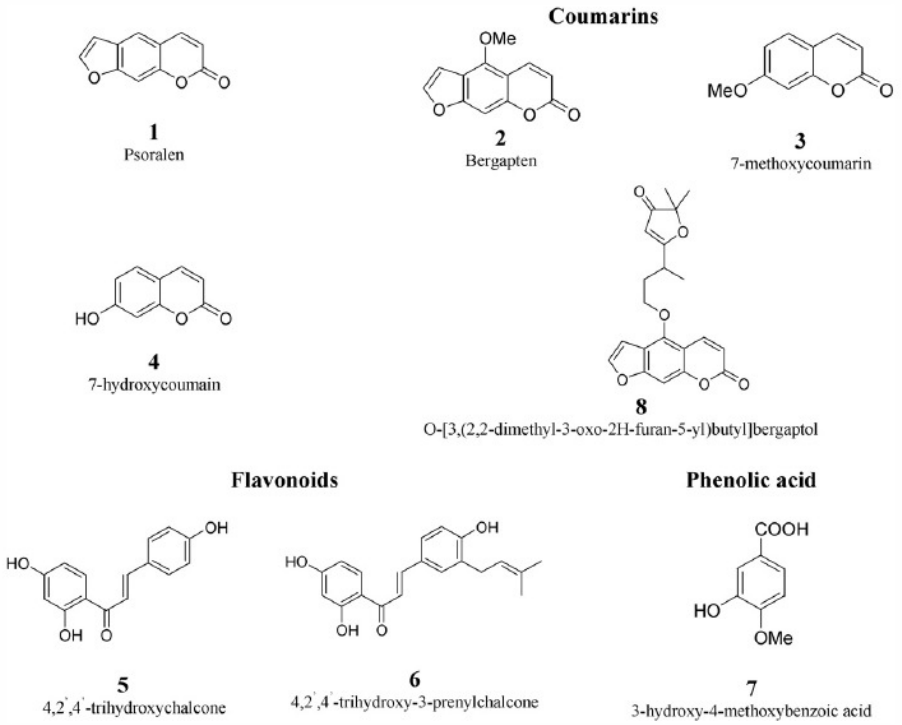

Fig. 1. Chemical structures of compounds isolated from the twigs of *Treculia obovoidea*.

(Zgoda and Porter, 2001; Kuete et al., 2007). The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37 °C for 24 h. The assay was repeated twice. Microbial growth was determined by observing the change of color in the wells (red when there is no growth and yellow when there is growth). The lowest concentration showing no color change was considered as the MIC.

For the determination of MMC, a portion of liquid (5 μl) from each well that showed no change in color was plated on MHA and incubated at 37 °C for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC.

3. Results and discussion

The structural identification of the compounds **1–8** were established using spectroscopic analysis, especially, NMR spectra in conjunction with 2D experiments, COSY, HMQC and HMBC, and direct comparison with published information and with authentic specimens obtained in our laboratory for some cases. The eight compounds isolated from *Treculia obovoidea* (Fig. 1) were found to belong to three secondary metabolites classes (coumarins, flavonoids, and phenolic acid). The coumarins (five) isolated from *Treculia obovoidea* were identified as Psoralen (**1**), Bergapten (**2**), 7-methoxycoumarin (**3**), 7-hydroxycoumarin (**4**) and *O*-[3-(2,2-dimethyl-3-oxo-2*H*-furan-5-yl) butyl]bergaptol (**8**). The flavonoids were found to be 4,2′,4′-trihydroxy-3-prenylchalcone (**6**), and 4,2′,4′-trihydroxychalcone (**5**) while the isolated phenolic acid was identified as 3-hydroxy-4-methoxybenzoic acid (**7**).

Many compounds from secondary metabolites classes such as coumarins, flavonoids, and phenolic acid have been reported from their antimicrobial activity (Cowan, 1999). The inhibition potential of compounds reported in this study is therefore in accordance with those studies.

The antibacterial and anticandidal activities of the crude extract and isolated compounds were evaluated and the results are reported in Tables 1 and 2. In general, there were differences in growth inhibition between compounds on various microbial cultures. The crude extract from *Treculia obovoidea* compounds **1** to **8** showed both antibacterial and anticandidal activities at the tested MIC limit of 156.25 μg/ml (Table 1). The MIC values obtained with the crude extract varied from 17 (80.95%) of the 21 tested microorganisms. For compounds isolated from TOT, the MIC values lower or equal to 156.25 μg/ml were obtained with compounds **5**, **6** and **9** on **14** (66.67%), compound **1** on **11** (52.38 %), compound **7** on **9** (42.86 %), compound **2** on **8** (38.10%), compound **4** on **7** (33.33%) and compound **3** on **4** (19.05%) of the tested microbial species. Compounds **5**, **6** and

Table 1
Minimum inhibition concentrations (μg/ml) of the methanolic extract, compounds isolated from the twigs *Treculia obovoidea* and reference antibiotics

Microorganisms	Tested samples[a]									
	TOT	1	2	3	4	5	6	7	8	RA[b]
Gram-negative bacteria										
Citrobacter freundii	78.12	78.12	–	–	–	19.53	19.53	156.25	9.76	4.88
Enterobacter aerogens	–	–	–	–	–	–	–	–	–	9.76
Enterobacter cloacae	156.25	39.06	78.12	–	–	19.53	9.76	–	9.76	4.88
Escherichia coli	156.25	–	–	–	–	–	–	–	–	1.22
Klebsiella pneumoniae	156.25	–	–	–	–	–	–	–	–	2.44
Morganella morganii	–	–	–	–	–	–	–	–	–	2.44
Proteus mirabilis	–	–	–	–	–	–	–	–	–	2.44
Proteus vulgaris	78.12	–	–	–	–	19.53	19.53	–	–	1.22
Pseudomonas aeruginosa	156.25	–	–	–	–	–	–	–	–	2.44
Shigella dysenteriae	–	–	–	–	–	–	–	–	19.53	2.44
Shigella flexneri	156.25	78.12	156.25	–	156.25	78.12	78.12	–	78.12	2.44
Salmonella typhi	156.25	–	–	–	–	9.76	4.88	–	78.12	1.22
Gram-positive bacteria										
Streptococcus faecalis	156.25	19.53	156.25	–	–	19.53	9.76	78.12	9.76	2.44
Staphylococcus aureus	156.25	–	–	–	156.25	19.53	9.76	–	19.53	4.88
Bacillus cereus	78.12	39.06	–	–	–	4.88	4.88	78.12	9.76	2.44
Bacillus megaterium	156.25	19.53	156.25	156.25	78.12	19.53	4.88	39.06	4.88	1.22
Bacillus stearothermophilus	156.25	19.53	–	–	–	19.53	9.76	39.06	9.76	4.88
Bacillus subtilis	78.12	9.76	78.12	–	78.12	19.53	4.88	39.06	9.76	2.44
Yeasts										
Candida albicans	78.12	19.53	78.12	19.53	39.06	19.53	4.88	19.53	4.88	2.44
Candida gabrata	156.25	39.06	78.12	78.12	78.12	19.53	39.06	78.12	9.76	2.44
Candida krusei	78.12	39.06	156.25	39.06	39.06	19.53	9.76	39.06	9.76	9.76

[a] Tested samples (TOT: methanolic extract from the twigs of *Treculia obovoidea*; Psoralen (**1**); Bergapten (**2**); 7-methoxycoumarin (**3**); 7-hydroxycoumarin (**4**); 4,2′,4′-trihydroxychalcone (**5**); 4,2′,4′-trihydroxy-3-prenylchalcone (**6**); 3-hydroxy-4-methoxybenzoic acid (**7**); *O*-[3-(2,2-dimethyl-3-oxo-2*H*-furan-5-yl)butyl]bergaptol (**8**)).
[b] RA: reference antibiotics (gentamycin for bacteria, nystatin for yeast); (–): MIC >156.25 μg/ml.

8 showed growth inhibition on all the six tested Gram-positive microorganisms. Regarding the degree of activity of compounds isolated from TOT, the lowest MIC value (4.88 μg/ml) was noted with compound **5** on *Bacillus cereus*, compound **6** on *Salmonella typhi*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis* and *Candida albicans*.

The results of the MMC determinations (Table 2) indicated that the MMC values lower than 156.25 μg/ml were observed with crude extracts on 39.29% (6/17) of the sensitive microbial species, respectively, for TOT. Within this tested interval (0.31–156.25 μg/ml), the MMC values were obtained with compounds on 9.52% (compound **3**) to 66.66% (compounds **5** and **6**) of the tested microorganisms.

When comparing the MIC interval of the antimicrobial activity of the tested samples to that of gentamycin (1.22–9.76 μg/ml) and nystatin (2.44–9.76 μg/ml) used as reference antibiotics, the inhibitory potency of tested compounds as well as that of the crude extracts could mostly be considered as important.

The results of the MMC determinations (Table 2) indicated that cidal effect of many of the tested sample could be expected. However, a keen look of the results of MIC (Table 1) and MMC (Table 2), showed that the MIC values obtained are four times lesser than the MMCs on corresponding (sensitive) microorganisms, confirming the microbicidal effects of the concerned samples (Carbonnelle et al., 1987).

To the best of our knowledge, the antibacterial and the anticandidal activities of *Treculia obovoidea* as well as that of compounds **6** and **8** is being reported for the first time. Nevertheless, this study supports the traditional use of plants of *Treculia obovoidea* in the treatment of infectious illness such as skin diseases, dysentery and AIDS (Berg et al., 1985; Bokesch et al., 2004). In addition, a good number of authors have documented the antimicrobial potency of some of the compounds isolated from *Treculia obovoidea* or their derivatives. Coumarins and coumarin-like compounds have been reported to possess antifungal activity (Benjamin and Hugbo, 1986). Céspedes et al. (2006) have also reported the antimicrobial activity of 7-methoxycoumarin (**3**) and 7-hydroxycoumarin (**4**). This study corroborated with these previous investigations. Furthermore, the antimicrobial properties of Psoralen (**1**) and Bergapten (**2**) are confirmed in the present study, as these compounds are used for the management of vitiligo, psoriasis and mycosis (Anderson and Voorhees, 1980).

Our investigation showed that the antimicrobial activity of the three *Treculia* genuses might be related to the presence of coumarin derivatives, flavonoids and phenolic acid. In fact, amongst the compounds isolated, the five coumarin-like compounds isolated from *Treculia obovoidea* exhibited a good anticandidal activity as compared with the reference substance (nystatin). Their good antibacterial activity was also observed.

Table 2
Minimum microbicidal concentrations (μg/ml) of the methanolic extract, compounds isolated from the twigs *Treculia obovoidea* and reference antibiotics

Microorganisms	Tested samples[a]									
	TOT	1	2	3	4	5	6	7	8	RA[b]
Gram-negative bacteria										
Citrobacter freundii	156.25	nd	–	–	–	39.06	78.12	nd	39.06	9.76
Enterobacter aerogens	–	–	–	–	–	–	–	–	–	19.53
Enterobacter cloacae	nd	78.12	156.25	–	–	39.06	19.53	–	39.06	9.76
Escherichia coli	nd	–	–	–	–	–	–	–	–	2.44
Klebsiella pneumoniae	nd	–	–	–	–	–	–	–	–	4.88
Morganella morganii	–	–	–	–	–	–	–	–	–	4.88
Proteus mirabilis	–	–	–	–	–	–	–	–	–	4.88
Proteus vulgaris	156.25	–	–	–	–	39.06	39.06	–	–	2.44
Pseudomonas aeruginosa	nd	–	–	–	–	–	–	–	–	4.88
Shigella dysenteriae	–	–	–	–	–	–	–	–	39.06	4.88
Shigella flexneri	nd	156.25	nd	–	nd	156.25	156.25	–	nd	4.88
Salmonella typhi	nd	–	–	–	–	39.06	9.76	–	156.25	2.44
Gram-positive bacteria										
Streptococcus faecalis	nd	39.06	nd	–	–	39.06	19.53	nd	19.53	4.88
Staphylococcus aureus	nd	–	–	–	nd	39.06	39.06	–	39.06	9.76
Bacillus cereus	156.25	78.12	–	–	–	19.53	9.76	156.25	39.06	4.88
Bacillus megaterium	nd	39.06	nd	nd	156.25	39.06	9.76	78.12	9.76	2.44
Bacillus stearothermophilus	nd	39.06	–	–	–	39.06	19.53	78.12	39.06	9.76
Bacillus subtilis	156.25	19.53	nd	–	156.25	39.06	9.76	78.12	19.53	4.88
Yeasts										
Candida albicans	156.25	39.06	156.25	39.06	156.25	39.06	9.76	39.06	9.76	4.88
Candida gabrata	nd	78.12	156.25	nd	156.25	78.12	78.12	156.25	39.06	4.88
Candida krusei	156.25	78.12	nd	78.12	78.12	39.06	39.06	78.12	19.53	19.53

[a] Tested samples (TOT: methanolic extract from the twigs of *Treculia obovoidea*; Psoralen (**1**); Bergapten (**2**); 7-methoxycoumarin (**3**); 7-hydroxycoumarin (**4**); 4,2′,4′-trihydroxychalcone (**5**); 4,2′,4′-trihydroxy-3-prenylchalcone (**6**); 3-hydroxy-4-methoxybenzoic acid (**7**); *O*-[3-(2,2-dimethyl-3-oxo-2*H*-furan-5-yl)butyl]bergaptol (**8**)].
[b] RA: reference antibiotics (gentamycin for bacteria, nystatin for yeast); (–): not tested because the MIC was not determined; nd: not determined because MMC > 156.25.

The flavonoids isolated from this species also exhibited a very good antibacterial and anticandidal activities.

As regards coumarins structure–activity relationships, it would appear that the additional furo-cycle generally increases the antimicrobial activity. However, the results of Table 1 showed that three cycle coumarins (**2**, **1** and **8** with the respective antimicrobial spectra of 38.10%, 52.38% and 66.67%), have more pronounced inhibition effects than coumarins with two cycles (**3** and **4** with the respective antimicrobial spectra of 19.05% and 33.33%). Within the three-cycle coumarins, the substitution of the 5-methoxy (–OCH_3) group of compound **2** by the *O*-[3-(2,2-dimethyl-3-oxo-2*H*-furan-5-yl) butyl]-group leading to compound **8** also increase the antimicrobial activity. For the phenolic acids such as compound **7**, it has also been demonstrated that the site and number of –OH groups on phenol group are related to their activity on microorganisms, with the evidence that increased hydroxylation results in increased toxicity (Gelssman, 1963).

The overall results of this study can be considered as very promising in the perspective of new drugs discovery from plant sources, if consider the medical importance of the tested microorganisms. *Pseudomonas aeruginosa* has emerged as one of the most problematic Gram-negative pathogens, with the alarmingly high antibiotics resistance rates (Bacq-Calberg et al., 1999; Savafi et al., 2005). Even with the most effective antibiotics against this pathogen, namely carbapenems (imipenem and meropenem), the resistance rates were detected as 15–20.4% amongst 152 *Pseudomonas aeruginosa* strains (Savafi et al., 2005). This pathogen was found to be sensitive to the crude extract. *Bacillus* species especially *Bacillus cereus* is agents of food poisoning (Avril, 1997; Sleigh and Timbury, 1998). *Salmonella typhimurium* is etiologically the most important agent of food toxi-infections (Avril et al., 2000). *Candida albicans* and other *Candida* species, causing candidiasis, are increasingly important diseases worldwide distributed, due to the fact that they are frequent opportunistic pathogen in AIDS patients (Cowan, 1999). The prevalence of the typhoid fever caused by *Salmonella typhi* is increasing in developing country nowadays. Generally, at least one sample tested in this study prevented the growth of each microbial strain.

The known antimicrobial mechanisms associated to each class of chemical to which the isolated compounds belong, may explain the antimicrobial potency of the crude extract and compounds from *Treculia obovoidea*. The interaction with eucaryotic DNA leading to growth inhibition (Cowan, 1999) could be the possible mechanism by which coumarin-like compounds (**1**, **2**, **3**, **4** and **8**) exhibit their anticandidal action. The activity of flavonoids such as compound **5** and **6** might be due to their ability to complex with bacterial cell wall (Cowan, 1999) and therefore, inhibiting the microbial growth. The mechanism

thought to be responsible for phenolic compounds toxicity (such as 7) to microorganisms includes enzyme inhibition by oxidized compounds, possibly through reaction with sulfhydryl groups or through more non-specific interactions with proteins (Mason and Wasserman, 1987).

Finally, the antimicrobial activity of the crude extract from *Treculia obovoidea* may be due to the presence of both antifungal and antibacterial compounds. The present study provides an important basis for the use of extracts from these plants for the treatment of infections associated to the studied microorganisms. The crude extract as well as the isolated compounds found active could be useful for the development of new antimicrobial drug. However, pharmacological and toxicity studies currently going on in our laboratory will be necessary to confirm this hypothesis.

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