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NMR Assignment and Antimicrobial/Antioxidant Activities of 1β-Hydroxyeuscaphic acid from the Seeds of *Butyrospermum parkii*

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Abstract – Phytochemical investigation of the EtOAc and *n*-BuOH fractions obtained from the crude methanol extract of the seeds of *Butyrospermum parkii* led to the isolation of seven compounds including 1β,2α,3α-trihydroxyurs-12-en-28-oic acid (1β-hydroxyeuscaphic acid) which full NMR assignment is herein reported for the first time. The above extract, fractions and some of the isolated compounds were screened for antimicrobial and antioxidant activities; 1β-hydroxyeuscaphic acid was the more potent.
Keywords – *Butyrospermum parkii*, Sapotaceae, Triterpenoids, Antimicrobial activity, Antioxidant activity

Introduction

Butyrospermun parkii Kotschy (*Butyrospermun paradoxum* (Don) Hepper) is a small tree that grows up to 14 m high, it belongs to the family Sapotaceae in which triterpenoids and saponins are fairly encountered (Wandji *et al*., 2003; Lavaud *et al.*, 1996). The seeds of this plant contain an edible fat (Kapseu *et al*., 2001) which is used in traditional medicine to treat scabies, ulcers, wounds and nasal stiffness (Ogunwande *et al*., 2001). Previous works on the shea butter (*B. parkii*) from the northern part of Cameroon revealed the presence of fatty acids (mainly oleic acid and stearic acid) and fourteen triglycerides detected by gas chromatography (Kapseu *et al*., 2001). Recently, Ogunwande *et al.* (2001) evaluated the anti-bacterial and antifungal properties of extracts from the leaves, stem bark, root bark, fruit and seed kernels of *B. parkii*. In our continuous search for new and/or known bioactive compounds from Cameroonian medicinal plants (Tapondjou *et al*., 2005a; Teponno *et al.*, 2006;Teponno *et al.*, 2007, Ponou *et al*., 2008), we have studied the methanol extract from the seed kernels of *B. parkii* growing in the western highlands of Cameroon. This article deals with the isolation and structural elucidation of seven compounds including 1β-hydroxyeuscaphic acid (**1**) which full NMR assignment is herein reported for the first time. The antimicrobial and antioxidant activities of the crude extract, fractions and isolated compounds were evaluated and the results obtained are presented.

Experimental

General – IR spectra were measured as a film on a KBr pellet using a FTIR-8400S Shimadzu spectrometer. ESI mass spectra were carried out on a Hewlett Packard HP-1100 series LC-MSD system. ^{1}H and ^{13}C NMR, DEPT, COSY, HSQC, HMBC and ROESY experiments were performed in deuterated MeOH on a Varian Mercury Plus Spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). All chemical shifts (δ) are given in ppm units with reference to tetramethylsilane (TMS) as the internal standard and the coupling constants (*J*) are in Hz. Column chromatography was performed using silica gel 60 Merck (63 - 200 μm and 32 - 63 μm). TLC was carried out on precoated Kieselgel 60 F_{254} (Merck) plates developed with CH_2Cl_2-MeOH (96-4; 92-8) and EtOAc-MeOH-H_2O (95-5-2). TLC plates were visualised by spraying with 50% H_2SO_4 and heating for 10 min at 110 °C.

Plant material – The seeds of *B. parkii* (Sapotaceae) were collected in Bangwa village, near the city of Bangangte (Western Region of Cameroon) in August 2005. Specimens documenting the collection were deposited

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in the Cameroon National Herbarium, Yaoundé (Ref: N° 58927/HNC).

Extraction and isolation – Dried and powdered seed kernels of *B. parkii* (2.5 kg) were extracted three times with MeOH at room temperature. The filtrate obtained was evaporated under reduced pressure to give a dark residue (258 g), which was suspended in water and successively partitioned with EtOAc and *n*-BuOH. The EtOAc fraction (10 g) was submitted to silica gel (70 - 200 µm) column chromatography eluted with the mixture Hexane-EtOAc in increasing order of polarity to give four main sub-fractions (A-D). Sub-fraction C (5 g) Hexane/ EtOAc (6 : 4 to 4 : 6) was repeatedly chromatographed on silica gel column eluted with CH_2Cl_2-MeOH (96 : 4) to give 1β-hydroxyeuscaphic acid (**1**, 30 mg), euscaphic acid (**2**, 10 mg), myrianthic acid (**3**, 25 mg) and (*E*)-*N*-(4-hydroxyphenethyl)-4-hydroxycinnamamide (**4**, 20 mg).

The *n*-butanol fraction (52 g) was dissolved in distilled water, poured into a Diaion HP-20 column. Initially water was used for elution to remove sugars and ionic substances and then 1.5 L MeOH was used and the resulted eluate was evaporated. A part of the MeOH residue (23 g) was subjected to a silica gel column chromatography eluted with EtOAc-MeOH (100 : 0 to 80 : 20), yielding four main sub-fractions. The sub-fraction eluted with EtOAc (2 g) was chromatographed on silica gel column using the mixture EtOAc-MeOH-H_2O (100 : 10 : 5) for elution to give 9,10,13-trihydroxyoctadec-11-enoic acid (**5**, 11 mg). Filtration of the sub-fraction eluted with EtOAc-MeOH (90 : 10) gave (8*E*)-*N*-2'-hydroxytetracosanoyl-1-*O*-D-glucopyranosyl-4-hydroxy-8-sphingogenine (**6**, 14 mg). The last sub-fraction (1 g) was rechromatographed on silica gel column eluted with CH_2Cl_2-MeOH (92:8) to give gallocatechin (**7**, 10 mg).

1β,2α,3α-trihydroxyurs-12-en-28-oic acid (1β-hydroxyeuscaphic acid) (**1**), white powder CH_2Cl_2-MeOH, IR (KBr) cm^{-1}: 3373, 2912, 1720, 1461, 1118; ^1H and ^{13}C NMR data: see Table 1; ESI-MS (pos.): *m/z* 527 $[M+Na]^+$ ($C_{30}H_{48}O_6$).

Microorganisms – The microorganisms used in this study consisted of three bacteria (*Staphylococcus aureus* ATCC25922, *Klebsiella pneumoniae* ATCC13883, *Salmonella typhi* ATCC6539) and two *Candida* species (*Candida albicans* ATCC9002 and *Candida tropicalis* ATCC750); all of which are reference strains obtained from the American Type Culture Collection. Also, included was one strain of *Cryptococcus neoformans* IP95026 obtained from the Pasteur Institute (IP, Paris-France). The bacterial and yeast strains were grown at 35 °C and maintained on nutrient agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) slants respectively.

Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) – MICs were determined by broth micro dilution method as described by Kuete *et al* with slight modifications. The test samples were first of all dissolved in dimethylsulfoxide (DMSO). The solution obtained was then added to Mueller Hinton Broth (MHB) for bacteria or Sabouraud Dextrose Broth (SDB) for yeasts to give a final concentration of 4000 µg/ml. This was serially diluted two fold to obtain concentration ranges of 4000 to 0.48 µg/ml. One hundred microliters of each concentration was added in each well (96- wells microplate) containing 95 µl of MHB or SDB and 5 µl of inoculum for final concentrations varying from 2000 to 0.24 µg/ml. The inoculum was standardized at 1.5×10^6 CFU/ml by adjusting the optical density to 0.1 at 600 nm JENWAY 6105 UV/Vis spectrophotometer. The final concentration of DMSO in each well was less than 1% (preliminary analyses with 1% (v/v) DMSO do not inhibit the growth of the test organisms). The negative control well consisted of 195 µl of MHB or SDB and 5 µl of the standard inoculum. The plates were covered with the sterile lid, then agitated to mix the contents of the wells using a plate shaker and incubated at 35 °C for 24 hours (for bacteria) or for 48 hours (for yeasts). The assay was repeated thrice. The MICs of samples were determined by adding 50 µl of a 0.2 mg/ml *p*-iodonitrotetrazolium chloride solution followed by incubation at 35 °C for 30 min. Viable micro-organisms reduced the yellow dye to a pink colour. MICs were defined as the lowest sample concentrations that prevented this change in colour indicating a complete inhibition of microbial growth.

For the determination of MMCs, a portion of liquid (5 µl) from each well that showed no growth of microorganism was plated on Mueller Hinton Agar or Sabouraud Dextrose Agar and incubated at 35 °C for 24 hours (for bacteria) or 35 °C for 48 hours (for yeasts). The lowest concentrations that yielded no growth after this sub-culturing were taken as the MMCs (Kuete *et al.*, 2008). Gentamicin (Sigma-Aldrich, Steinheim, Germany) and Nystatin (Merck, Darmstadt, Germany) for bacteria and yeasts respectively were used as positive controls.

Antioxidant assay: DPPH assay method – The free radical scavenging activity of the crude extract as well as their isolated compounds was evaluated as described by Mensor *et al.* (2001) with slight modifications. The test samples, prior dissolved in DMSO (SIGMA) were mixed with a 20 mg/l 2,2-diphenyl-1-picryl-hydrazyl-hydrate

(DPPH) methanol solution, to give final concentrations of 10, 50, 100, 500 and 1000 μg/ml. After 30 min at room temperature, the absorbance values were measured at 517 nm and converted into percentage of antioxidant activity as follows:

% inhibition = [(Absorbance of control – Absorbance of test sample)/Absorbance of control] × 100. Ascorbic acid was used as a standard control. The inhibition ratio was converted in probits. The probit values were plotted against the logarithmic values of concentrations of the test samples and a linear regression curve was established in order to determined the IC_{50} (μg/ml) ; being the amount of sample necessary to decrease by 50% the absorbance of DPPH. All the analyses were carried out in triplicate and the results were expressed as the mean ± standard deviation (SD) and compared using Waller-Duncan test. A value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Compound **1** was isolated as a white powder in MeOH. Its ESI-MS (positive-ion mode) exhibited a pseudo-molecular ion peak at m/z 527 $[M+Na]^+$ consistent with the molecular formula of $C_{30}H_{48}O_6$. It showed IR absorptions bands at 3373 (OH), 2912 (CH) and 1720 (C=O) cm^{-1}. The ^1H NMR spectrum revealed the presence of seven methyl groups (six singlets and one doublet) and a vinylic proton signal at δ 5.32 (brs, H-12) suggesting an ursane triterpenoid derivative with one oxidized methyl (Wandji *et al*., 2003; Lavaud *et al*., 1996). This spectrum

Table 1. NMR data for compound 1, CD_3OD, *J* (Hz) in brackets

Position	^{13}C (δ)	^1H (δ)	HMBC
1	79.8 (d)	3.43 (d,11.4)	C-25, C-5, C-2
2	70.4 (d)	3.63 (dd, 4.4, 11.4)	C-1
3	79.2 (d)	3.40 (d, 4.4)	C-5, C-2, C-1
4	37.6 (s)	–	
5	48.1 (d)	2.01 (nd)	C-6, C-9, C-10, C-3, C-7, C-25
6	18.1 (t)	1.62 (m)	
7	32.7 (t)	nd	
8	40.4 (s)	–	
9	23.8 (d)	2.43 (m)	
10	41.1 (s)	–	
11	48.4 (t)	3.31	C-18; C-19
12	129.3 (d)	5.32 (brs)	C-10; C-9; C-18
13	137.4 (s)	–	
14	41.6 (s)	–	
15	27.7 (t)	2.01 (nd)	C-13
16	22.6 (t)	1.38 (nd)	
17	48.2 (s)	–	
18	53.5 (d)	2.48 (s)	C-14; C-17; C-19; C-12; C-13; C-28
19	72.2 (s)	–	
20	42.9 (d)	1.38 (m)	
21	28.2 (t)	0.90 (m)	C-22; C-29
22	37.5 (t)	2.57 (m)	C-21; C-18
23	16.3 (q)	0.78 (s)	C-6
24	21.1 (q)	0.83 (s)	C-5; C-3
25	11.6 (q)	1.02 (s)	C-10; C-1
26	27.8 (q)	nd	
27	25.9 (q)	1.20 (s)	C-14
28	180.9 (s)	–	
29	25.2 (q)	1.38 (s)	
30	15.3 (q)	0.85 (d, 7.0)	C-29; C-19; C-20;

nd: not determined

also exhibited signals at δ 3.43 (d, J = 11.4 Hz, H-1), 3.63 (dd, J = 4.4, 11.4 Hz, H-2), 3.40 (d, J = 4.4 Hz, H-3) corresponding to three oxygenated methines (Table 1). The ^{13}C NMR data of **1** were similar to those of 2α,3α-dihydroxyurs-12-en-28-oic acid (euscaphic acid) **2** previously isolated from the fruits of *Fragaria ananassa* (strawberry) (Hirai *et al*., 2000). Nevertheless, there was an obvious difference, in fact an up-field methylene resonance in **1** was absent and replaced by a newly arisen signal at δ 79.8 (C-1), suggesting that the methylene group was hydroxylated in **1**. The HMBC correlations observed between H-1 (δ 3.43) and C-2 (δ 70.4) , H-2 (δ 3.63) and C-1 (δ 79.8), H-3 (δ 3.40) and C-2 (δ 70.4), H-3 (δ 3.40) and C-1 (δ 79.8) and between H-18 (δ 2.48) and C-19 (δ 72.2) confirmed that compound **1** was a 19α-hydroxyurs-12-ene derivative with a 1,2,3-trihydroxylated A-ring. Many other correlations were also observed (Table 1). The ROESY correlations between H-2 and H-3, H-2 and H-23, and between H-2 and H-25 (Fig. 2) combined with the large coupling constant J = 11.4 Hz for H-1 indicated the β orientation of the hydroxyl group at C-1. Consequently, the structure of **1** was elucidated as 1β,2α,3α-trihydroxyurs-12-en-28-oic acid (1β-hydroxy-euscaphic acid) previously isolated from *Rosa sterilis* (Guang-Yi *et al*., 1999) and its full NMR assignment is herein reported for the first time.

The comparison of spectroscopic and physical data of the other compounds with those published in the literature allowed us to identify them as euscaphic acid **2** (Guang-Yi *et al*., 1999), myrianthic acid **3** (Wandji *et al*., 2003), (*E*)-*N*-(4-hydroxyphenethyl)-4-hydroxycinnamamide **4** (Chang *et al*., 2001), 9,10,13-trihydroxyoctadec-11-enoic acid **5** (Mats, 1991), (8*E*)-*N*-2'-hydroxytetracosanoyl-1-*O*-β-D-glucopyranosyl-4-hydroxy-8-sphingogenine **6** (Tapondjou *et al*., 2005b; Yamauchi *et al*., 2001) and gallocatechin **7** (Seto *et al*., 1997).

The MeOH extract, *n*-BuOH and EtOAc fractions as well as some isolated compounds were tested for their anti-microbial activity. The results obtained are presented in Table 2. The crude MeOH extract has shown both antibacterial and antifungal activities, on the set of germs tested with MIC values varying from 62.5 to 1000 µg/ml. The EtOAc fraction was more active (MIC = 31.25 – 250 µg/ml) than the *n*-butanol fraction (MIC = 125 – 2000 µg/ml) and the crude extract (MIC = 62.50 – 1000 µg/ml), this means that the fractionation must has increased the activity of the EtOAc fraction and reduced that of the *n*-BuOH fraction; thus suggesting that the active principles might be more concentrated in the EtOAc fraction.

Compound **1** isolated from the EtOAc fraction was more active (MIC = 3.90 – 62.5 µg/ml) than all the other

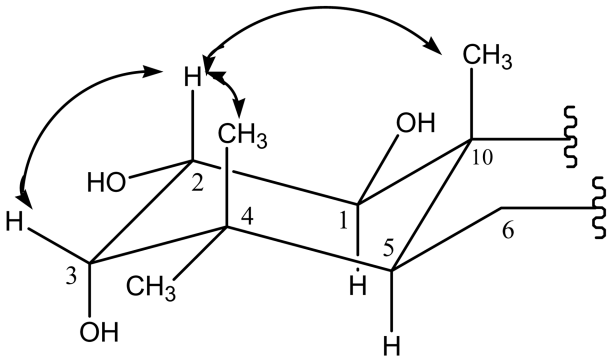

Fig. 2. Some important ROESY correlations for compound **1**.

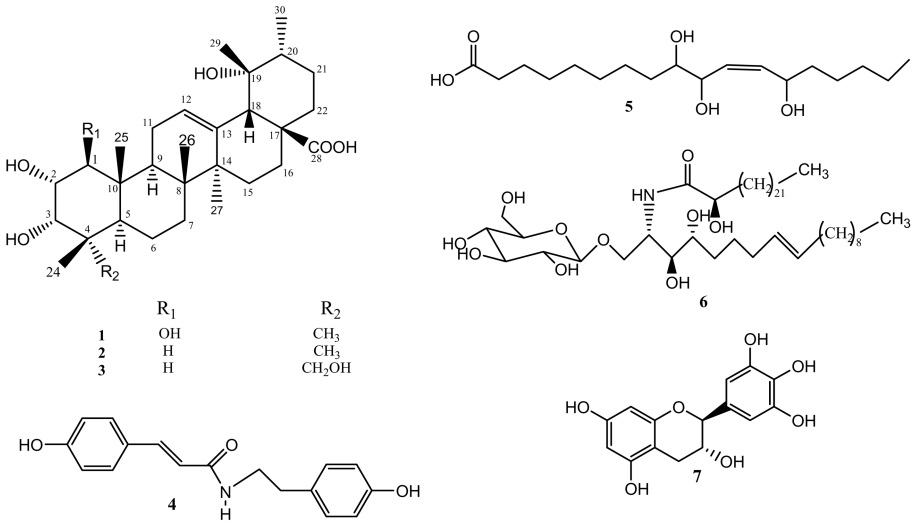

Fig. 1. Structures of compounds **1**-**7** isolated from the seed kernels of *B. parkii*.

Table 2. Antimicrobial activity (in µg/ml) of the crude methanol extract (ME), ethyl acetate fraction (EAF), *n*-butanol fraction (nBF) and some isolated compounds

Test samples	*Inhibition parameters*	*Salmonella typhi ATCC 6539*	*Staphylococcus aureus ATCC 25922*	*Klepsiella pneumoniae ATCC 13883*	*Candida albicans ATCC 9002*	*Cryptococcus neoformans IP 95026*	*Candida tropicalis ATCC 750*
	MIC	500	125	1000	1000	250	62.50
ME	MMC	500	125	2000	1000	500	125
	MIC/MMC	2	1	2	1	2	2
	MIC	62.50	31.25	125	250	125	31.25
EAF	MMC	62.50	31.25	500	250	125	31.25
	MIC/MMC	1	1	4	1	1	1
	MIC	250	250	2000	2000	500	125
nBF	MMC	250	500	2000	2000	1000	250
	MIC/MMC	1	2	1	1	2	2
	MIC	62.50	31.25	>500	>500	250	62.50
7	MMC	125	31.25	/	/	250	62.50
	MIC/MMC	2	1	/	/	1	1
	MIC	500	250	>500	>500	>500	>500
2	MMC	>500	>500	/	/	/	/
	MIC/MMC	/	/	/	/	/	/
	MIC	15.62	7.81	15.25	62.50	3.90	3.90
1	MMC	31.25	7.81	15.25	125	7.81	3.90
	MIC/MMC	2	1	1	2	2	1
	MIC	>500	125	>500	>500	31.25	15.62
3	MMC	/	125	/	/	31.25	15.62
	MIC/MMC	/	1	/	/	1	1
	MIC	>500	500	>500	>500	>500	>500
6	MMC	/	500	/	/	/	/
	MIC/MMC	/	1	/	/	/	/
	MIC	62.50	7.81	125	>500	125	31.25
4	MMC	62.50	15.62	125	/	250	62.50
	MIC/MMC	1	2	1	/	2	2
	MIC	31.25	31.25	15.25	/	/	/
Gentamicin	MMC	31.25	31.25	15.25	/	/	/
	MIC/MMC	1	1	1	/	/	/
	MIC	/	/	/	1.95	1.95	7.81
Nystatin	MMC	/	/	/	1.95	1.95	7.81
	MIC/MMC	/	/	/	1	1	1

/ : not determined

tested compounds. It is followed in decreasing order by compounds **4**, **7**, **3**, **2** and **6**. The antibacterial activity of compound **1** was generally higher than that of the positive control gentamicin. Nevertheless, the antifungal activity of this compound (MIC = 3.90 – 62.5 µg/ml) was less than that of the positive control nystatin (MIC = 1.95 – 7.81 µg/ml).

Compound **1** has the same basic skeleton as compounds **2** and **3** but their antimicrobial activities are different. The presence of a hydroxyl group at position C-1 may have increased the antimicrobial activity of compound **1** (Fig. 1). In comparison with compound **2**, compound **3** with the same basic skeleton was more active. The presence of the hydroxyl group at position C-23 in compound **3** could be responsible for the difference in the observed antimicrobial activity. The antimicrobial properties of some individual triterpenes of plant origin are documented (Barre *et al*., 1997; Djoukeng *et al*.,

Table 3. DPPH radical scavenging activities of the test samples (IC_{50}).

Test samples	IC_{50} (µg/ml)
Crude MeOH extract (ME)	54.95 ± 0.67[a]
EtOAc fraction (EAF)	85.11 ± 0.31[b]
n-butanol fraction (nBF)	38.01 ± 1.23[c]
7	21.37 ± 0.46[d]
1	18.62 ± 0.58[e]
4	23.44 ± 0.74[f]
Vit. C	18.62 ± 0.33[e]

Values are expressed as mean ± SD. In the same column, values affected by the different superscript letters (a-f) are significantly different at $p < 0.05$.

2005). It was also found that MMC values obtained were generally less than four fold greater than the MICs (Table 2) on the corresponding microbial species, suggesting that a cidal effect of the crude extract and some the isolated compounds could be expected on most of the tested organisms (Carbonnelle *et al*., 1987; Mims *et al*., 1993). This is interesting in the perspective of developing new antimicrobial drugs from the tested samples.

The crude MeOH extract, the two fractions and some of the isolated compounds were also evaluated for their antioxidant activity using DPPH assay method (Fig. 3 and Table 3). The inhibition percentage of free radical (DPPH) increases with the concentration of the tested substances (Fig. 3). The MeOH extract (IC_{50} = 54.95%) was more active than the EtOAc fraction (IC_{50} = 85.11%) but less than the n-butanol fraction (IC_{50} = 38.01%) (Table 3). The antimicrobial activity was more concentrated in the EtOAc fraction and contrarily, the antioxidant activity is more concentrated in *n*-butanol fraction. The antioxidant activity of compound **1** (IC_{50} = 18.62%) was comparable to that of the reference vitamin C (IC_{50} = 18.62%); while no activity was noticed for compounds **2**, **3** and **6** (not shown). The non activity of compounds **2** and **3** and the high activity of **1** indicated that the location of the OH group at C-1 may be very important for the antioxidative activity in the triterpene skeleton. Several oxygenated pentacyclic triterpenes have also been reported as antioxidant compounds (Garcez *et al*., 2006; Pawar and Bhutani, 2005; Montilla *et al*. 2003). Phenolic compounds **4** and **7** are known to be potential antioxidant due to their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicals (Hall and Cuppett, 1997; Pietta *et al*., 1998). Therefore, the presence of these compounds could be responsible for the antioxidant activity found in the crude extract and fractions.

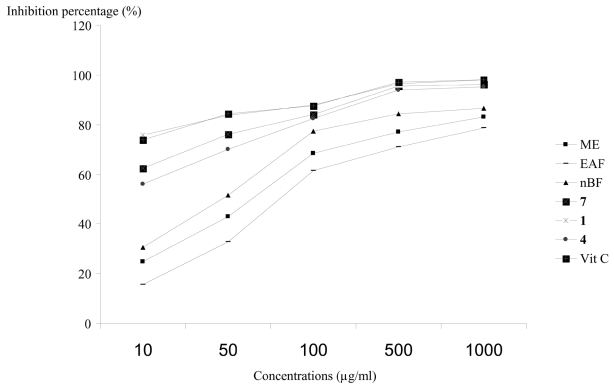

Fig. 3. Free radical scavenging activity of the crude MeOH extract (ME), EtOAc fraction (EAF), *n*-butanol fraction (nBF) and some of the isolated compounds (**1**, **4** and **7**).

The present study clearly demonstrated that *Butyrospermum parkii* possesses antimicrobial and antioxidant properties and therefore it might reduce the risk of aging-related diseases and/or promote general human health.

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