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Antioxidant and antimicrobial activities of chemical constituents from *Celtis australis*

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ABSTRACT

The chemical investigation of the hydro-methanolic extract of leaves from Celtis australis led to the isolation of 3 known compounds: 2 sterols (β-sitosterol and β-sitosterol-3-O-β-glucoside) and 1 phenolic acid (vanillic acid) which are reported for the first time in this plant species. Antioxidant activity of the isolated compounds was assessed by in vitro measurement of DPPH (1, 1'-diphenyl-2-picrylhydrazil) radical scavenging activity and conjugated dienes (CD) and TBARS (thiobarbituric acid reactive substances) inhibition during linoleic acid peroxidation. Antimicrobial activity of isolated compounds was also carried out by the quantification of minimum inhibitory concentration (MIC) using disc diffusion technique against seven bacterial and three fungal strains.
The results of the present study showed the significant (P<0.01) antioxidant and antimicrobial activities of the tested compounds.

Keywords: Antimicrobial, Antioxidant, *Celtis australis*, chemical constituents

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INTRODUCTION

Cannabaceae is a large family, containing about 15 genera and 200 species. The largest genus, Celtis, includes about 60 species. Among these species is *Celtis australis*, commonly known as European nettle tree, Mediterranean hackberry or honeyberry. It is a deciduous tree endemic to southern Europe, North Africa, and south-western Asia [1, 2]. *Celtis australis* is widely used in many traditional systems as medicinal plant. In Indian traditional medicine, the decoction of both leaves and fruits is used in the treatment of amenorrhea, heavy menstrual and inter-menstrual bleeding, diarrhea, dysentery and peptic ulcers [3]. The paste obtained from the bark of *C. australis* is considered as an important remedy for bone fracture and also applied on pimples, contusions, sprains and joint pains [4]. In Moroccan traditional medicine, *C. australis* commonly called "Taghzaz" is mainly used to treat gastro-intestinal ailments [5].

Because of their traditional medicinal applications, various Celtis species have been studied for their chemical constituents, leading to the isolation of steroids [6-8], flavonoid-glycosides [9-13], phenolic amids [6, 14], sulphonated phenolics and bacteriohopanoid [7, 15], terpenoids [16, 6, 8], anthraquinone [8], glucosphingolipid [17], tannins, saponins and alkaloids [18], fatty acids [19], apigenin, quercetin and its glucoside [8].

In a continuation of our previous studies on *Celtis australis* [20-22], we report herein the isolation and chemical structures of 3 known compounds from this plant for the first time and their antioxidant and antimicrobial properties.

MATERIALS AND METHODS

Plant Material

The leaves of *C. australis* were collected from El Jadida city (Morocco) in July 2014. The plant was identified and authenticated at the laboratory of botany at the biology department. A voucher specimen (reference CA10/13) is kept on file in our laboratory.

Extraction and isolation

The air-dried leaves (2.5Kg) of *Celtis australis* were powdered by an electrical mill mesh and sieved using a fine muslin cloth. The obtained powder was exhaustively extracted by maceration using methanol-eau mixture (3V/2V) for 3 days at room temperature and repeated three times. The crude solution was dried by rotavapor to yield 308.6g of a dark green sticky paste. It was then dechlorophyllated according to the method described by Herz and Hogenaner [23] giving 117.2g of crude extract. About 20 g of the crude extract, dissolved in a minimum amount of ethanol, were subjected to flash chromatography on silica gel column and successively carried out with the mixtures of chloroforme-petroleum ether (V/V; 7V/3V; 9V/1V), chloroforme-acetone (9V/V; 7V/3V; 3V/7V) and acetone in increasing order of polarity. The eluates were collected in 25 ml portions and combined upon monitoring by TLC giving three major fractions:

Fraction A was concentrated giving whitish syrup which on placing in ethyl acetate followed by recrystallization in chloroforme afforded 15.9 mg of compound 1.

Fraction B (748mg), chromatographied on silica gel column with chloroforme-petroleum ether mixtures (1V/9V; 3V/7V; V/V, 9V/1V) in increasing polarity, gave 89mg of compound 2.

Fraction C was subjected to repeated column chromatography on a silica gel column with the mixture of chloroforme-acetone (9V/V; 7V/3V; 3V/7V) in increasing polarity. The sub-fraction, corresponding to eluates 229-233, precipitated white granules weighing 30 mg of compound 3.

Spectral analysis

The ^{1}H and ^{13}C-NMR spectra, including DEPT 135 technique, were recorded in $CDCl_3$ as internal standard on Bruker Aspect AMX-500 NMR instrument operating at 500 MHz. The chemical shifts were recorded in ppm (δ) and coupling constants (J) in Hz.

Antioxidant tests

DPPH radical scavenging activity

The DPPH free radical is a stable molecule widely used to assess radical scavenging activity (RSA) of antioxidant compounds. The method is based on the reduction of DPPH radical in methanol solution in the presence of a hydrogen–donating antioxidant due to the formation of the non-radical form DPPH-H. This transformation results in a color change from purple to yellow, which is monitored spectrophotometrically at 517nm.

The RSA of the tested compounds was evaluated according to the modified method previously reported by Blois [24]. A solution of DPPH (0.1mM) in methanol was prepared and 2.5ml of this solution was mixed with each tested compound or BHT (positive control) at final concentrations of 4, 8 and 16µg/ml. The reaction mixture was vortexed and left in the dark at room temperature. The absorbance of the control (A_c) and samples (A_s) was measured, and the RSA, expressed in percentage, was calculated as follow:

$$\text{RSA } (\%) = \frac{A_c - A_s}{A_c} \text{x} 100$$

Measurement of conjugated dienes (CD)

During the formation of hydroperoxides throughout the peroxidation of lipids, conjugated dienes (CD) are typically produced, due to the rearrangement of the double bonds. The resulting CD exhibit an intense absorption at 234 nm. Thus, the increase in UV absorption reflects the formation of CD, the primary oxidation products of lipids peroxidation, with good correlation between CD and peroxidation process.

CD generation was measured spectrophotometrically at 234 nm according to the modified method described by Esterbauer et al. [25]. A mixture of linoleic acid (7.5 mM), emulsified with Tween 20 (0.1 v/v per cent), in phosphate buffer (pH 7.4), at a final concentration of 10 mM, was incubated alone (control) or with each compound at final concentrations of 4, 8 and 16µg/ml. The oxidation was initiated by the addition of freshly prepared $CuSO_4$,

at a final concentration of 10 µM. It was stopped by cooling in an ice bath, in the presence of EDTA (100 µM) and BHT (20 µM). BHT was used as positive control at final concentrations of 4, 8 and 16µg/ml.

The inhibition of CD production was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} x100$$

Where:
A_c = Absorbance of control
A_s = Absorbance of the sample

Measurement of TBARS
TBARS are naturally present in biological systems and include lipid hydroperoxides and aldehydes that increase in concentration as response to oxidative stress of biological molecules. The quantification of TBARS was monitored, according to the modified method as described by Ohkawa et al. [26]. A mixture of linoleic acid (7.5 mM), emulsified with Tween 20 (0.1 v/v per cent), in phosphate buffer (pH 7.4), at a final concentration of 10 mM, was incubated at 37°C alone (control) or with each compound at final concentrations of 4, 8 and 16µg/ml. The oxidation was initiated by the addition of freshly prepared $CuSO_4$, at a final concentration of 10 µM. It was stopped by cooling in an ice bath, in the presence of EDTA (100 µM) and BHT (20 µM). TBARS were measured by addition of 1ml of trichloracetic acid (20%) and 1ml of thiobarbituric acid (0.78%) to each sample. The mixtures were boiled in water bath during 45min. The reaction was stopped by cooling in an ice bath and the TBARS extracted with n-butanol. The absorbance of the sample supernatant (A_s) was readied at 532nm against absorbance of control (A_c) that contained all reagents except linoleic acid. The inhibition of TBARS production was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} x100$$

BHT was used as standard at final concentrations 4, 8 and 16µg/ml.

Antimicrobial test
Microorganisms
The antibacterial screening was conducted against four Gram positive bacteria (*Staphylococcus aureus*, *Bacillus sp*, *Bacillus cereus* and *Listeria ivanovii),* and three Gram negative bacteria (*Escherichia coli*, *Citrobacter freundii* and *Salmonella sp).*

For antifungal test, two yeasts (*Candida albicans* and *Candida tropicalis*) and one filamentous fungus (*Aspergillus niger*) were used.

All the microorganisms were procured from Pasteur Institute (Casablanca, Morocco). They were maintained by subculturing periodically and stored at +4°C prior to use. For inocula preparations, bacteria and yeast strains were incubated for 24 h in Mueller Hinton Agar (MHA) medium and filamentous fungus for 3 days in Potato Dextrose Agar (PDA).

Quantification of minimum inhibitory concentrations (MICs)
MICs of the isolated compounds were determined by tube dilution method (turbimetric method). The microbial cultures were diluted in broth at a density corresponding to a 0.5 McFarland turbidity standard corresponding to 10^8 CFU/ml for bacterial strains and 10^5 CFU/ml for fungal strains. Each compound was serially diluted between1/2 to 1/512 giving respective concentrations from 500 to 1.9mg/ml. To 180µl of microbial suspension was added 20µl of each tested compound at every concentration, and then incubated under aerobic conditions for 24h (bacterial strains) or 48h (fungal strains) at 37°C or 28°C respectively. Negative control samples contained sterile nutrient broth. Tetracycline and Fluconazole were used, in the same conditions, as positive controls. The lowest concentration that did not allow any noticeable bacterial growth when compared with the negative control was considered as the minimum inhibitory concentration (MIC). All the experiments were performed in triplicate and the results expressed as mean value ± standard deviation.

Statistical analysis
The experimental results were expressed as mean ± standard deviation (SD) of three replicates. Where applicable, the data were subjected to one way analysis of variance (ANOVA). P Values < 0.01 were considered as significant.

RESULTS AND DISCUSSION

Phytochemical investigation

Compound 1 was crystallized from chloroforme as white crystalline powder (15.9 mg). It responded to Liebermann-Burchard reaction indicating a steroidal substance. It gave negative Molisch test. The Rf value in chloroforme-petroleum ether (9V/1V) was 0.65.

The ^{1}H NMR of compound 1 exhibited methyl signals at δ: 0.67 (Me-18), 0.81 (Me-26), 0.83 (Me-27), 0.84 (Me-29), 0.91 (Me-21), and 1.02 (Me-19). This compound has revealed one proton multiplet at 3.50 (H-3). The shift at 5.35 was evident for the olephinic hydrogen (H-6). From DEPT it is appeared that compound 1 contained 29 carbons: six methyl carbon (–CH_3), eleven methylene carbon (-CH_2-), nine methine carbon (>CH-) and three quaternary carbons (>C<) with a hydroxyl group. Methyl carbons (C-18, C-19, C-21, C-26, C-27, and C-29) appeared at δ 11.99, 19.41, 18.78, 19.84, 19.03, 11.87 ppm; methylene carbons (C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23, and C-28) appeared at δ 37.25, 31.66, 42.30, 31.92, 21.09, 39.17, 24.31, 28.26, 33.94, 26.26 and 23.06 ppm; methine carbons (C-3, C-6, C-8, C-9, C-14, C-17, C-20, C-24, and C-25) appeared at δ 71.82, 121.73, 31.90, 50.12, 56.78, 56.04, 36.15, 45.82 and 29.13 ppm; and quaternary carbons (C-5, C-10, and C-13) appeared at δ 140.76, 36.51 and 42.32 ppm. By correlating our data with those of literature [27-29], compounds 1 was identified as β-sitosterol (figure 1).

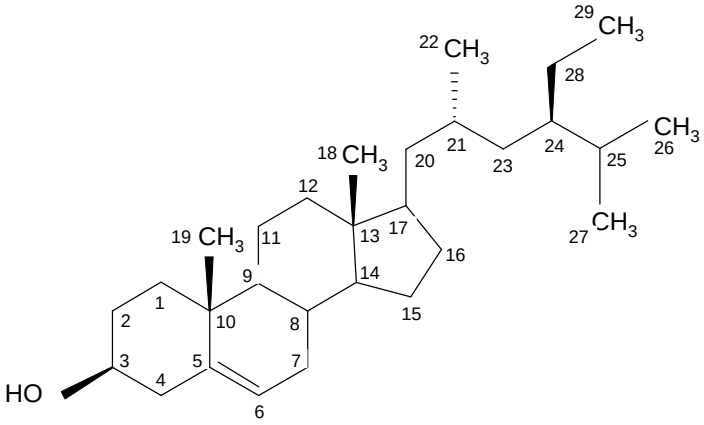

Figure 1: Chemical structure of β-sitosterol

Compound 2 was crystallized from chloroforme as white crystalline powder (89mg), mp 280°C. It responded to Liebermann-Burchard reaction indicating a steroidal substance. It gave positive Molisch test indicating the presence of glucidique fraction. The Rf value in chloroforme-petroleum ether (9V/1V) was 0.55. The ^{1}H NMR spectrum of compound 2 showed six shift at δ 0.65, 0.96, 0.90, 0.82, 0.81 and 0.83 ppm for methyl hydrogen (-CH_3) at positions 18, 19, 21, 26, 27 and 29 respectively. One proton at C-3 appeared as multiplet at 3.42 ppm and a doublet at 5.32 ppm was the characteristics of double bond in the ring between quaternary carbon C-5 and methine carbon C-6. These at δ 4.90, 4.87, 4.87, 4.43, 3.50 and 4.21 can be attributed to protons at positions 1', 2', 3', 5' and 6' in sugar moiety. The ^{13}C NMR exhibited 6 peaks at δ 11.75, 19.69, 18.58, 18.89, 19.69 and 11.64 ppm for the six methyl group at positions 18, 19, 21, 26, 27, and 29. Those of methylene carbons appeared at δ 36.79, 31.34, 39.19, 33.30, 22.55, 38.26, 25.36, 29.22, 39.44, 27.77 and 23.83 ppm that were correlated to positions C-1, C-2, C-4, C-7, C-11, C-12, C-15, C-16, C-22, C-23, and C-28 respectively. The methine carbons C-3, C-6, C-8, C-9, C-14, C-17, C-20, C-24, and C-25 appeared at δ 70.04, 121.19, 31.38, 49.56, 55.38, 56.14, 35.45, 45.09 and 28.64 ppm. The quaternary carbons (C-5, C-10, and C-13) appeared at δ 140.4, 35.93 and 45.68 ppm. The glucose unit contained six carbons of which oxygenated carbon C-1 appeared at 100.73 ppm and methylene carbon C-6 appeared at 61.04 ppm. The other four carbons of the glucose molecule were appeared at 70.04, 73.42, 76.72 and 76.84 ppm. The similarity of our data with those of literature [30, 31], led us to identify compound 2 as β-sitosterol-3-O-β-glucoside (Figure 2).

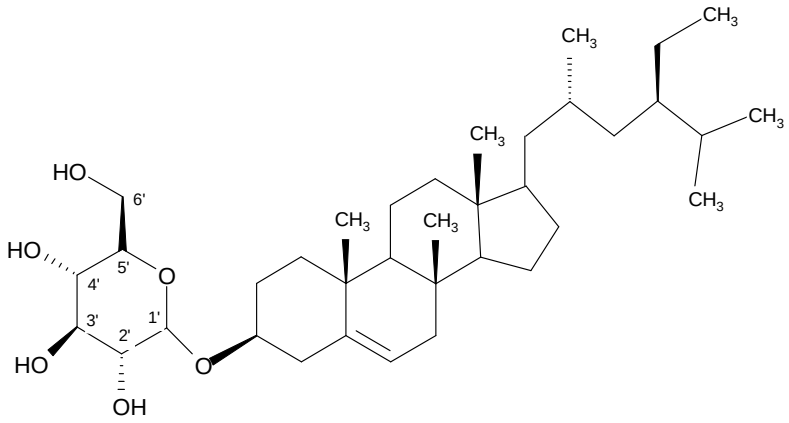

Figure 2: Chemical structure of δ-sitosterol-3-O-δ-glucoside

Compound 3 was crystallized from acetone as white crystalline powder (30mg).The Rf value in chloroforme-acetone (3V/7V) was 0.55. The data of ^{1}H NMR were given as follow δ: 7.58 (H, d, j=1.9Hz, H-2), 6.97 (H, d, J=8.3 Hz, H-5), 7.71 (H, dd, J=8.3, 1.9 Hz, H-6) and 3.97 (3H, s, CH_3O). Those of ^{13}C NMR were summarized as follow δ : 121.1 (C-1 and C-2), 145.2 (C-3), 150.7 (C-4), 114.2 (C-5), 125.1 (C-6), 169.8 (COOH) and 56.1 (CH_3O). By correlating our data with those of literature [28, 32], compounds 1 was identified as vanillic acid (figure 3).

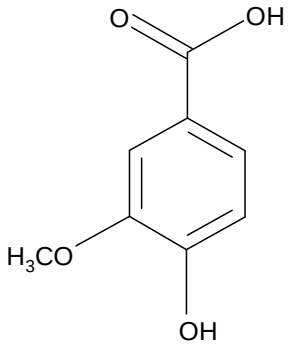

Figure 3: Chemical structure of vanillic acid

Antioxidant activity

The isolated compounds were tested for their antioxidant potential using three different methods. i) DPPH test, which enables to measure the radical scavenging activity, ii) Quantification of conjugated dienes which allows to estimate the antioxidant activity of extracts during primary lipid oxidation phase and iii) TBARS method which evaluates the antioxidant properties of extracts during secondary lipid oxidation phase. The quantification of different oxidation products, at different stages of the oxidative process, provides more detailed information about this dynamic pathway [33].

DPPH radical scavenging activity

As shown in figure 4, all the tested compounds showed a significant ($P<0.01$) and dose-dependent scavenging effect on the DPPH radical. At 16µg/ml, vanillic acid showed the highest activity among all the tested samples (75%), followed by β-sitosterol-3-O-β-glucoside (66 %) and β-sitosterol (64%) which were higher than that produced by BHT (58%). Otherwise, as shown in table 1, the IC_{50} values of tested compounds corroborated their highest radical scavenging activity with regard to BHT. Vanillic acid was the highest active (8.2µg/ml), followed by β-sitosterol-3-O-β-glucoside (9.5µg/ml) and β-sitosterol (10µg/ml) whereas the one of BHT was 12µg/ml. It is well known that the effect of scavengers on DPPH is thought to be due to their electron accepting/hydrogen donating abilities. The tested compounds proceed probably with this mechanism to quell the free radicals, acting possibly as primary antioxidants. This assumption is corroborated by Vivancos and Moreno [34] who showed that the GSH and GSH/total glutathione ratio recovered after treatment by β-sitosterol suggesting that this phytosterol could be a ROS scavenger. In the same way several studies suggested that the antioxidative potency of phenolic acids, such as vanillic acid and their derivatives, depended on the number of hydroxyl groups in the molecules and on the stabilization of the radical formed via an increased electron delocalization [35, 36].

Figure 4: Radical scavenging activity (RAS) of Vanillic acid (VA), β-sitosterol-3-O-β-glucoside (BSG) and β-sitosterol (BS) isolated from *C. australis*

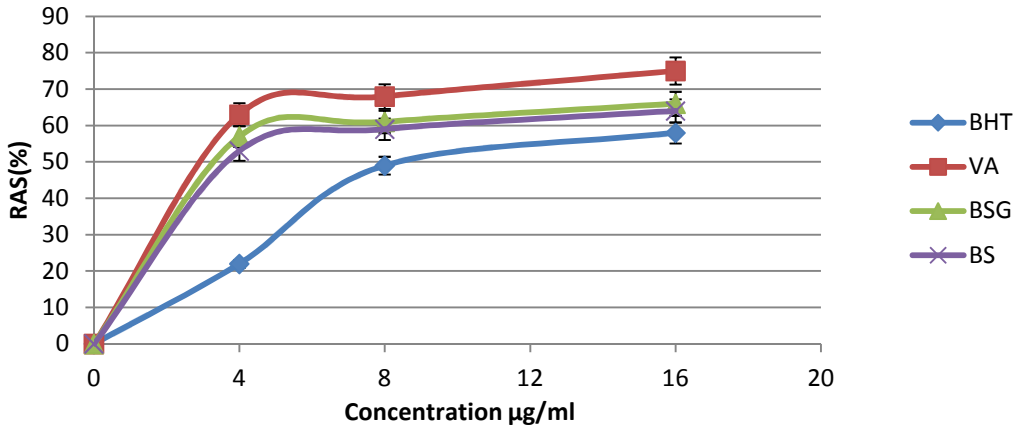

Table 1: IC_{50} of DPPH radical scavenging activity of isolated compounds from *C. australis*

Isolated compounds	IC_{50} µg/ml
Vanillic acid	8.2
β-sitosterol-3-O-β-glucoside	9.5
β-sitosterol	10.0
BHT	12.0

Otherwise, our results are corroborated by several reports which have shown a significant radical scavenging capacity of phytosterols, mainly β-sitosterol and β-sitosterol 3-O-β-glucoside [27, 37, 38] in one hand and vanillic acid in the other hand [39-41].

Conjugated dienes (CD) inhibition

Polyunsaturated fatty acids, such as linoleic acid, are easily oxidized. This oxidation leads to the occurrence of chain reactions with the formation of coupled double bonds primary oxidation products such as conjugated dienes [25]. In order to estimate the antioxidant potential of the isolated compounds during primary oxidation process, the inhibition of the peroxidation of the linoleic acid was quantified at various concentrations (4, 8, and 16µg/ml). As shown in figure 5, a significant ($P<0.05$) and dose-dependent inhibitory effect was obtained on CD production during linoleic acid oxidation in solution. At 16µg/ml, all the tested samples showed a significant inhibitory effect ($P<0.01$) on CD production which decreased in the order of vanillic acid> β-sitosterol-3-O-β-glucoside>β-sitosterol that were higher than the one exhibited by BHT. The values of IC_{50} reported in table 2, showed high antioxidant potential of the tested compounds with regard to BHT. The antioxidant effect of phytosterols was established by several studies. Thus, Ferreti et al. [42] have investigated the in vitro interactions between β-sitosterol, campesterol, and stigmasterol and LDL isolated from normolipemic subjects. The authors demonstrated that these phytosterols exert an inhibitory effect against copper-induced lipid peroxidation of LDLs, as shown by the lowered levels of CD in oxidized LDL incubated with different concentrations of plant sterols (5–50 mM). These phytosterols prevent also the alterations of apoprotein structure and physicochemical properties associated with copper-triggered lipid peroxidation of lipoproteins. The antioxidant property of phytosterols was also showed in vivo by previous studies which suggested that these molecules counteract oxidative stress through the modulation of antioxidant enzymes and free radical production [34]. In the same way, Da Silva Marinelli et al. [44] reported the reduction of hepatic lipid peroxidation products and plasma levels of MDA (malondialdehyde) in rats after phytosterols supplementation. Otherwise, the antioxidant potential of vanillic acid was also showed by Kumar et al. [45] who reported the significant reduction of the levels of TBARS, LOOH (lipid hydroperoxides) and CD in L-arginine-methyl ester hydrochloride-treated rats.

Figure 5: Inhibition of conjugated dienes (%) by Vanillic acid (VA), β-sitosterol- 3-O-β-glucoside (BSG)and β-sitosterol (BS) isolated from *C. australis*

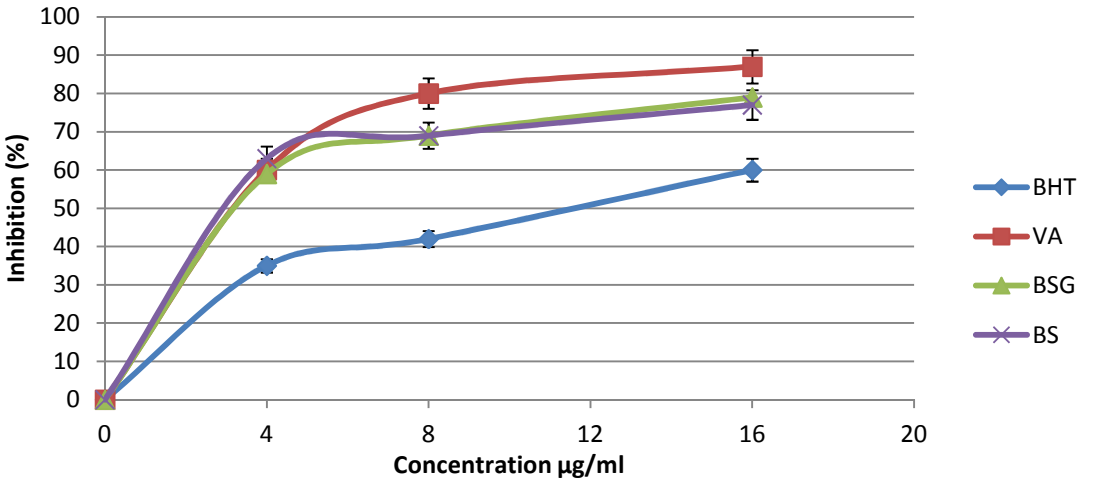

Table 2: IC_{50} of conjugated dienes inhibition of isolated compounds from *C. australis*

Isolated compounds	IC_{50} µg/ml
Vanillic acid	7.4
β-sitosterol-3-O-β-glucoside	8.1
β-sitosterol	8.1
BHT	11.5

TBARS inhibition

During the process of lipid peroxidation, hydroperoxide is produced as the first stable product in both the radical and non-radical reactions, and TBARS are the secondary products, which are expressed as MDA, an indicator of cell oxidative stress. Figure 6 reported the inhibitory effect of the tested compounds on TBARS production, indicating a significant anti-lipid peroxidation activity ($P<0.01$). At 16µg/ml, the inhibition percentages were in the range of 95, 80 and 72% for vanillic acid, β-sitosterol, and β-sitosterol-3-O-β-glucoside respectively. In the same conditions, effect exerted by BHT was weak and reached 70%. The IC_{50} values of the samples were 7, 8.5, and 9µg/ml for vanillic acid, δ-sitosterol, and β-sitosterol-3-O-β-glucoside respectively, whereas the one of BHT was 10µg/ml (table 3). Several findings suggested that phytosterols were responsible, at least in part, for preventive effects on the development of diseases due to reactive oxygen species. Yoshida and Niki [46] reported the antioxidant effects of δ-sitosterol, stigmasterol, and campesterol against lipid peroxidation. Van Rensburg et al. [47] showed that low concentrations of β-sitosterol and other sterols reduced levels of TBARS, secondary products of lipid peroxidation, while high concentrations of β-sitosterol promoted the increase of this marker in vitro. Antioxidant activity has also been attributed to these plant compounds in foods, animal models and human studies [48-50].

Figure 6: TBARS inhibition by Vanillic acid (VA), β-sitosterol-3-O-β-glucoside (BSG) and β-sitosterol (BS)isolated from *C. australis*

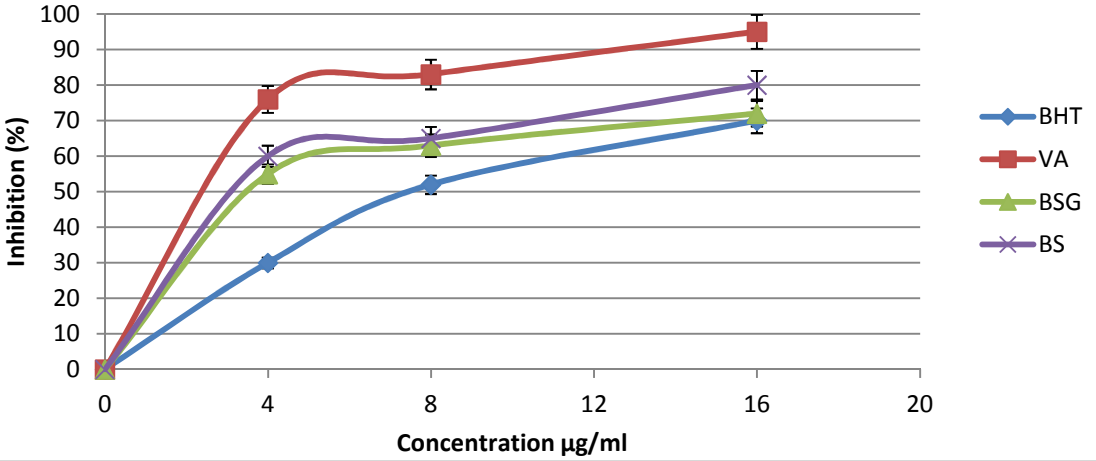

Table 3: IC_{50} of TBARS inhibition of isolated compounds from *C. australis*

Isolated compounds	IC_{50} µg/ml
Vanillic acid	7.0
β-sitosterol-3-O-β-glucoside	9.0
β-sitosterol	8.5
BHT	10.0

It is well known that lipid peroxidation is caused, at least in part, by molecules having unpaired electrons. Antioxidant compounds are molecules that can accept electron/donate hydrogen. These include ring structures as found in steroid molecules, electron acceptors such as oxygen, as well as glucose. This could explain how the phytosterols tested in this study inhibited lipid oxidation.

Otherwise, the protective effect of vanillic acid, on lipid peroxidation by reducing MDA formation, was also showed by Chou et al. [51] who suggested the possible role of carboxyl group constituent, which can transfer electrons /donate protons, and act as a primary and secondary antioxidant in the inhibition of lipid peroxidation processes.

Antimicrobial activity

The values of MICs are summarized in Table 4. All the tested compounds showed significant antibacterial activity ($P<0.01$) against all bacterial strains, albeit to varying extent. Very strongest activity (MIC= 25µg/ml) was observed with vanillic acid against *B. cereus*. Strongest activity (MIC=50µg/ml) was recorded with vanillic acid against *B. sp*, *C. freundii* and *S. sp*, and with β-sitosterol-3-O-β–glucoside against *B. sp*. Moderate activity (MIC = 100µg/ml) was obtained with vanillic acid against *L. ivanovii, S. aureus*, and *E. coli*, with β–sitosterol 3-O-β-glucoside against *L. ivanovii, C. freundii* and *S. sp* and with δ-sitosterol against *B. sp*. Weak activity (MIC=200µg/ml) was showed with β-sitosterol-3-O-β-glucoside against *B. cereus, S. aureus*, and *E. coli* and with β-sitosterol against *B. cereus, L. ivanovii, E. coli*, and *S. sp*. Tetracycline showed very strongest activity (MIC=12.5 to 25 µg/ml) against 2 bacterial strains (*L. ivanovii, and S. aureus*), strongest activity (MIC=50µg/ml) against 4 bacterial strains (*B. sp, B. cereus, C. freundii* and *S. sp*) and moderate activity (MIC=100µg/ml) against *E. coli*.

Table 4: Antibacterial activity of isolated compounds from *C. australis*

	Strain	MICs µg/ml			
		Vanillic acid	β-sitosterol 3-O-β–glucoside	β-sitosterol	Tétracycline
Gram+	*B. sp*	50	50	100	50
	B. cereus	25	200	200	50
	L. ivanovii	100	100	200	12.5
	S. aureus	100	200	100	25
Gram-	*C. freundii*	50	100	ND	50
	E. coli	100	200	200	100
	S. sp	50	100	200	50

Table 5 reported the MICs values of the isolated compounds against 3 fungal strains. *C albicans* was sensitive (MIC=50µg/ml) to vanillic acid and β–sitosterol-3-O-β-glucoside, whereas *C. tropicalis* was sensitive toward vanillic acid and δ-sitosterol. Moderate antifungal activity (MIC=100µg/ml) was showed with vanillic acid against *A. niger*, with β-sitosterol-3-O-β-glucoside against *C. tropicaliss* and *A. niger* and with β–sitosterol against *C. albicans* and *A. niger*. Otherwise, all tested fungal strains were very sensitive to Fluconazol.

Table 5: Antifungal activity of isolated compounds from *C. australis*

Strain	MICs µg/ml			
	Vanillic acid	β-sitosterol 3-O-β-glucoside	β-sitosterol	Fluconazol
C. albicans	50	50	100	12.5
C. tropicalis	50	100	50	6.25
A. niger	100	100	100	12.5

Several studies have reported the antimicrobial activity of different phytosterols corroborating our results. Thus, Subramaniam et al. [52] showed that β-Sitosterol-glucoside have significant antimicrobial activity against *S. aureus, B. subtilis, E. faecalis, E. coli, V. cholera, K. pneumoniae, P. vulgaris, P mirabilis, S. dysenteriae* and *P. aeruginosa* (MICs=6-50 µg/ml). Furthermore, the time kill curves showed that this compound kills most of the pathogens within 5-10 h**.** Sen et al. [53] established that β-sitosterol at 20µg/ml has antimicrobial activity against *E. coli, P. aeruginosa, S. aureus* and *K. pneumoniae* almost equivalent to that of Gentamycin at the same dose. Kiprono et al. [54] reported the antibacterial effect of δ-sitosterol from *Senecio lyratus* against *S. typhii* and *C. diphtheria* and its

antifungal activity against *Fusarium spp*. However, the antimicrobial potential of phytosterols have not established by some studies. Thus, Bayor et al. [55] reported no antibacterial activity of β–sitosterol and β-sitosterol-3-O-β-glucoside isolated from *Croton membranaceus* against *S. aureus, B. subtilis* and *P. aeruginosa*. In the same way, β-sitosterol isolated from *Coccoloba acrostichoides* exhibited no effect against *S. aureus, M luteus, A. niger* and *F. Oxysporum* [56].

The antimicrobial activities of vanillic acid reported in this study were corroborated by other researches which demonstrated its bacteriostatic and bactericidal effects against seven *Cronobacter spp* [57]. Aziz et al. [58] reported the antibacterial effect of Oleuropein, and vanillic, p-hydroxybenzoic, and p-coumaric acids mixture (0.4mg/ml) against *E. coli, K. pneumoniae* and *B. cereus*, and the antifungal activity of vanillic and caffeic acids mixture (0.2 mg/ml) against *A. flavus* and *A. parasiticus*, The antifungal activity of vanillic acid was also showed by De Souza et al. [59] against *L. gongylophorus*.

CONCLUSION

To the best of our knowledge, until this moment, it is the first time that β-sitosterol, β-sitosterol-3-O-β-glucoside and vanillic acid are being cited as constituents of *C. australis*. All these compounds exhibited in vitro free radical scavenging effect and antioxidant activity during linoleic acid peroxidation, assessed by CD and TBARS production, when compared with BHT as standard antioxidant. The in vitro antioxidant potency of tested compounds is probably due to their capacity to scavenge radicals by transfer electrons/donate protons, and act as a primary and secondary antioxidant in the inhibition of lipid peroxidation process. The tested compounds showed also related antimicrobial and antifungal properties with regard to Tetracycline and Fluconazol used as antibiotic standards. Although these results corroborated the utilization of *C. australis* in traditional medicine, important questions remain regarding the in vivo efficacy of the tested compounds. Further scientific investigations and clinical trials are required to support these results and establish their therapeutic efficacy.

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