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Investigating Antioxidant Properties of the Diterpenes from Seeds of *Phalaris canariensis*

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Abstract

Aims: The role of free radical reactions in disease pathology is well established, suggesting that these reactions are necessary for normal metabolism but can be detrimental to health as well acting on different levels in an oxidative sequences, the antioxidants prevents the risk of several aging related diseases including cancer, cardio vascular disorder, diabetes, neurodegenerative disorders and others.

Main methods: Bioactivity-guided fractionation of a hexane extract of the seeds of *P. canariensis* afforded three new diterpenes: Canarien A-C, their structures were established based on interpretation of their MS, as well as 1D and 2D NMR data. In this study, investigating the antioxidant activities of Canarien A-C, which have been evaluated in assay •OH, $•O_2^-$, DPPH•, HOCl•, NO, ONOO•, H_2O_2, ORAC, SOD, $ABTS^+$-scavenging assays, Fe^{2+}-chelating, lipid peroxidation, inhibition lipoxygenase (LOX) and determination of enzymatic antioxidants activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH**),** and glutathione peroxidase (CSH-Px) in liver.

Key findings: The three diterpenes showed effective Hdonor, free radical scavenging, metal chelating ability, and inhibition of lipid peroxidation compared to standard antioxidants such as BHT, α-tocopherol, curcumin, BHA and quercetin. In addition showed inhibition of lipoxygenase and a moderate increase in the activity of enzymes SOD, CAT, GSH and CSH-Px in liver.

Significance: It may be concluded that hexane extract and diterpenes from *P. canariensis* seeds acts as an antioxidant and ROS scavenger, may protect against oxidative stress and hepatic damages.

Keyword: *Phalaris canariensis;* Antioxidant capacity; Diterpenes; Abietane; Pimarene

Introduction

The aging of human organs, cancer, immune dysfunction, and other diseases are closely related to the oxidative damage in cells induced by free radicals, and antioxidants play an important role in inhibiting and scavenging free radicals, thus it can provide protection for human beings. Synthetic antioxidants are commercially available but quite unsafe, and their toxicity is a worrying problem. The natural antioxidants, especially phenolics and flavonoids, are safe and they possess biological activity, so the current researcher is now directed toward natural antioxidants from natural plants [1]. The most promising strategy to prevent from the oxidative damage caused by these reactive species is the use of antioxidant molecules. These compounds can act as direct antioxidants through free radical scavenging mechanisms and/or as indirect antioxidants by enhancing the antioxidant status (enzymatic and non-enzymatic). Terpenes, one of the most extensive and varied structural compounds occurring in nature, display a wide range of biological and pharmacological activities. Due to their antioxidant behaviour terpenes have been shown to provide relevant protection under oxidative stress conditions in different diseases including liver, renal, neurodegenerative and cardiovascular diseases, cancer, diabetes as well as in ageing processes [2]. ROS are responsible for the damage of cellular bio-molecules such as proteins, enzymes, nucleic acids, lipids and carbohydrates and may adversely affect immune functions. Antioxidants interrupt the production of ROS and a1so play a key role to inactivate them. Although, all human cells protect themselves against oxidative damage by some antioxidant mechanism, these sometimes are not sufficient to prevent the ROS damage totally.

Canary seed is solely used as food for caged and wild birds. However the unique composition and characteristics of canary seed make it a promising cereal for food and industrial uses. In the past, canary seed was not seen as a viable cereal for human consumption due to the harmful effects associated with the siliceous hairs that cover the hull of the seed. These hairs are highly irritating when they come in contact with human skin or lungs and have been linked to esophageal cancer (Mukherjee, 2003). The variety was developed based on mutagenesis and traditional breeding by which a totally hairless variety was developed. Removing the damaging hairs rediscovered canary seed as a potential food crop and industrial crop for fractionation industry. The canary seed or alpiste, *Phalaris canariensis* L. is a member of a family of grasses (Grarninaceae), and it is used in folk medicine in the form of tea as a coadjuvant in the treatment of hypertension, diabetes mellitus, and hypercholesterolernia [3]. In Mexico the seeds of *Phalaris canariensis* are used as food human and birds also to have been traditionally used in folk medicine to treat various ailments and diseases. Some of these diseases involve reactive oxygen species (ROS) that can be prevented by the consumption of antioxidants compounds that can be found in plants [4]; however, such use has no scientific basis. There is one study related to the hypotensive effect of *P. canariensis* seed infusion in

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normotensive rats [5]. In previous studies we report the antiobesity and antidiabetic effects of the hexane extract from *Phalaris canariensis* [6]. The aim of the present study was to establish antioxidant activities of the three terpenes isolated from seeds of *Phalaris canariensis.*

Materials and Methods

General experimental procedures

IR spectra were obtained on a Perkin-Elmer 1720 FTIR. A Bruker DRX-300 NMR spectrometer, operating at 599.19 MHz for ^{1}H and 150.86 MHz for ^{13}C, using the UXNMR software package, was used for NMR experiments; chemical shifts are expressed in δ (ppm) using TMS as an internal standard. DEPT ^{13}C, ID TOCSY, LH-LH DQF-COSY, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature (Nolis and Parella, 2005). HREIMS were measured on a JEOL HX 110 mass spectrometer (JEOL, Tokyo, Japan). Precoated TLC silica gel 60 F254 aluminium sheets from Sigma-Aldrich (St. Louis, USA) were used. Column chromatography was carried out on Silica gel 60 (230-400 mesh, Merck Co. New Jersey, USA); solvents used as eluents were from Fermont (California, USA).

Plant materials and extraction

Seeds of *P. canariensis* were collected in Morelos State, Mexico. A voucher specimen (No. 8054) was deposited in the Herbarium of the National School of Biological Sciences, for further reference. Seeds powdered (10 kg) were exhaustively extracted with hexane, (40l) at room temperature. Crude extract, after filtering and evaporation of the solvent a viscous residue (423.8 g) was obtained. It was fractioned by CC (Si gel, 240-400 μm, column 460 x 36 mm) eluting with CH_2Cl_2 to give 8 fractions (A1-A8). Each fraction (75 ml) was monitored by (thin layer) TL; fractions with similar TLC patterns were combined to yield four major fractions. Each fraction was monitored for its antioxidant effect. Subfraction A8 was subjected to silica gel CC with mixture of chloroform/ petroleum ether (2:1) to afford 10 subfractions (subfracciones A8-1 - A8-10). Subfraction A-83 which was successively partitioned with a step gradient of petroleum ether/chloroform/ methanol (1 :0:0, 3:1:0, 1:1 :0, 1:3:0, 0:1:0, 0:99:1, 0:49:1, 0:97:3, 0:19:1, 0:97:7, 0:91:9, 0:89:11, 0:87:13 y 0:17:3) to obtain compound 1 (210.7 mg). Subfraction A-88 was was further separated by prep. TLC [Si gel, $CHCl_3$] and then purified on a Sephadex (LH-20) column using methylene chloride/hexane (2:0.5) to give compound 2 (186.5 mg) and compound 3 (181.2 mg).

Canarien A (1) pale yellow oil: IR νmax 3424 (OH), 3032, 2917, 1650 (aromatic), 1750, 1238 (acetate) cm^{-1}; $[\alpha]^{20}$ +23.4 (c 0.15, MeOH); EIMS m/z (rel. int.) 344.2372 (calc for $C_{22}H_{32}O_3$, 344.2351); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data shown in Table 1.

Canarien B (2) pale yellow oil: IR νmax 2997, 1736 (γ-lactone), 1730 cm^{-1} (acetyl group), 1238 (carbonyl) and 1642 (C=C) cm^{-1}; $[\alpha]^{20}$ +31.6 (c 0.15, MeOH); EIMS m/z (rel. int.) 360.2382 (calc for $C_{22}H_{32}O_4$, 360.2301); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data shown in Table 1.

Canarien C (3) pale yellow oil: IR νmax 2917, 1650 (C=C), 1738 (γ-lactone), 1731 cm^{-1} (acetyl group), 1238 cm^{-1} ; $[\alpha]^{20}$ + 40.6 (c 0.15, MeOH); EIMS m/z (rel. int.) 360.2362 (calc for $C_{22}H_{32}O_4$, 360.2301); ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data shown in Table 1.

Total phenolic compound analysis

The amount of total phenolic was determined with the Folin-Ciocalteu´s reagent using the method of Lister and Wilson (2001). To 50 μl of each sample (three replicates), 2.5 ml 1/10 dilution of Folin-Ciocalteau's reagent and 2 ml of Na_2CO_3 (7.5%, w/v) were added and incubated at 45°C for 15 min. The absorbance of all samples was measured at 765 nm in a Shimatzu-1800. Results were expressed as mg of gallic acid equivalent per g of dry weight (mg GAE/g dw).

Determination of free radical scavenging and antioxidant properties

DPPH scavenging assay: The DPPH scavenging assay was determined using the method described by Blois (1958). A 5 μM of compound or control was mixed with 450 μl PBS (10 mM/l, pH 7.4) and 1.0 ml of ethanolic DPPH (0.1 mM/l) solution. After a-30 min reaction, the absorbance was recorded at 517 nm.

Measurement of total antioxidant activity

The total antioxidant activity (TAA) values were estimated by the Trolox equivalent antioxidant capacity (TEAC) assay [7]. We measured using ABTS antioxidant Kit (Zen-Bio, Inc (Durham, North Carolina, USA). Results are presented as the ability of terpenoids to scavenge 50% of free radical ABTS•+ (IC_{50}) and TEAC (Trolox equivalent antioxidant capacity).

Total antioxidant activity by ferric reduced antioxidant power assay (FRAP): Experiments were done according to Benzie et al. [8] with modifications. FRAP working solution was prepared freshly each time: 0.3 M acetate buffer (pH=3.6), 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) in 0.04 M HCl and 0.02 M $FeCl_3.6H_2O$ were mixed in 10:1:1 v/v/v and kept away from light. 0.075 ml of diterpenes (final concentration 0.01 mg/m1) or $FeSO_4$. $7H_2O$ (final concentration 0-1.8 μM Fe^{2+}/m1) solution were added to 2.25 mi FRAP working solution and 0.225 ml of deionized water. The mixture was vortexed and incubated at 37°C for 30 min away from light. Absorbance was measured at 593 nm using the spectrophotometer Shimatzu-1800. FRAP working solution with deionized water instead of a sample was used as a blank. All determinations were carried out in triplicate.

HOCl Scavenging assay

The hypochlorous acid (HOCl•) scavenging activity was tested using the modification of the ferrocyanide [Fe(II)CN)6] oxidation method [9]. The reaction mixture solution containing 100 μl of samples and 450 μl of HOCl (0.4 mM) was incubated for 5 minutes at room temperature. After addition of 450 μl of ferrocyanide (20 mM), the resulting mixture was incubated for 5 minutes at room temperature, and the absorbance was measured at 420 nm using a spectrophotometer Shimatzu-1800.

Peroxynitrite scavenging activity

A previously described standard Choi et al. [10] method was followed to synthesize peroxynitrite ($ONOO^-$). 5 ml 0.6 M KNO_2 was mixed with an acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) on ice bath for 1 min and 5 ml of ice-cold 1.2 M NaOH was added to the solution. The solution was subjected to treatment with granular MnO_2 prewashed with 1.2 M NaOH to remove the excess H_2O_2. The reaction mixture was left overnight at -20°C. Peroxynitrite solution was collected from the top of the frozen mixture and the concentration was measured spectrophotometrically at 302 nm (ε=1670 M^{-1} cm^{-1}). To measure peroxynitrite scavenging activity an Evans Blue bleaching assay was used. The assay was performed by a standard method with a slight modification (Bailly et al., 2000). The reaction mixture contained

No	δ_H (*J* in Hz)	δ_c	δ_H (*J* in Hz)	δ_c	δ_H (*J* in Hz)	δ_c
1α	1.22, m	38.05	0.99, m	39.56	4.22, dd, 11.1, 5.0	69.87
1β	1.88, m	-	1.82, m	-	-	-
2α	1.96, m	35.67	1.62, m	20.12	1.99, m	21.62
2β	2.04, m	-	1.75, m	-	1.68, m	-
3α	4.68, dd, 11.5, 5.0	76.32	1.45, m	32.67	1.36, m	39.51
3β	-	-	1.88, m	-	1.27, m	-
4	-	53.1	-	42.18	-	-
5	2.21, brd, 12.3	53.76	1.50, d, 11.5	60.01	1.60, m	60.31
6α	1.60, m	20.31	4.21, t, 11.5	76.11	4.22, t, 11.5	76.18
6β	1.87, m	-	-	-	-	-
7α	2.92, m	30.42	-	69.98	2.06. m	34.56
7β	2.80, m	-	4.15, t, 3.0	-	2.67, m	-
8	-	131.56	2.56, m	38.17	1.58, m	38.32
9	-	142.73	1.22, m	37.19	1.26, m	36.93
10	-	38.67	-	35.98	-	36.1
11α	7.29, d, 8.5	127.92	1.90, m	20.32	1.94, m	19.03
11β	-	-	1.70, m	-	1.68, m	-
12α	7.17, d, 8.5	126.12	1.78, m	36.71	1.74, m	35.78
12β	-	-	1.50, m	-	1.59, m	-
13	-	144.21	-	36.23	-	35.63
14α	6.85, s	129.48	1.68, bd, 13.5	39.19	1.70, bd, 13.5	40.11
14β	-	-	1.59, d, 13.5	-	1.60, d, 13.5	-
15	3.05, sept, 7.0	34.52	5.90, dd, 10.6, 17.8	146.12	5.91, dd, 10.6, 17.8	146.24
16	0.87, d, 7.0	28.03	4.78, *cis*: dd, 5.11, d, 10.6 4.92, trans: 5.15, d, 17.8	112.43	4.77, *cis*: dd, 5.11, d, 10.6 4.92, trans: 5.15, d, 17.8	111.56
17	0.87, d, 7.0	28.03	0.83, s	24.12	0.88, s	26.43
18	1.29, s	23.59	-	179.74	-	177.9
19	4.07, d,11.6 3.56, d, 11.5	68.32	1.21, s	16.73	0.93, s	17.32
20	1.25, s	25.48	0.86, s	18.76	0.90, s	18.75
CH_3CO	2.12, s	21.32	2.16, s	22.01	2.09, s	21.98
CH_3CO	-	167.45	-	170.33	-	172.43

Table 1: ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data for compounds 1-3 (δ in ppm).

50 mM phosphate buffer (pH 7.4), 0.1 mM DTPA, 90 mM NaCl, 5 mM KCl, 12.5 μM Evans Blue, various doses of plant extract (0–200 μg/ml) and 1 mM peroxynitrite in a final volume of 1 ml. The absorbance was measured at 611 nm after incubation at 25°C for 30 min. The percentage scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. All tests were performed six times.

Singlet oxygen scavenging assay

The assay was performed according to previously reported spectrophotometric method with minor modifications (Pedraza-Chaverri et al., 2004). The production of singlet oxygen ($^1O_2^-$) was determined by monitoring the bleaching of N,N-dimethyl-4-nitrosoaniline (RNO). Singlet oxygen was generated by a reaction between NaOCl and H_2O_2 and the bleaching of RNO was read at 440 nm. The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2, 50 mM L-histidine, 10 μM RNO and various concentrations (0–100 μg/ml) of sample in a final volume 2 ml. The solution mixture was incubated at 30°C for 40 min and decrease in the absorbance of RNO was measured at 440 nm.

Oxygen radical absorbance capacity assay (ORAC)

Assay was performed per the kit OxiSelect™ Oxygen Radical Antioxidant Capacity (ORAC) Activity Assay (Cell Biolabs Inc. San Diego, USA).

Ferrous ion chelating ability

The method of Decker and Welch was used to investigate the ferrous ion chelating ability of compounds [11]. The reaction mixture containing 1.0 ml of different concentrations of the compounds (50-800 μg/ml) was mixed with 3.7 ml of methanol, 0.1 ml of 2 mM ferrous chloride and 0.2 ml of 5 mM ferrozine to initiate the reaction and the mixture was shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution was measured at 562 nm. The percentage chelating effect on ferrozine-Fe^{2+} complex was calculated.

Nitric oxide radical scavenging assay

This assay was performed according to the method described by Sreejayan et al. [12]. The reaction mixture (3 ml) containing 10 mM nitroprusside in phosphate buffered saline, and the fractions or the extracts at different concentrations (50-800 μg/ml) were incubated at 25°C for 150 min. About 0.5 ml aliquot incubated sample was removed at 30 min intervals and 0.5 ml Griess reagent was added. The absorbance of chromosphoro formed was measured at 546 nm.

Assay for inhibition effect on lipid peroxidation

A solution of 2 mg β-carotene in 10 ml chloroform and 1.0 ml of this solution was then pipetted to 20 mg of linoleic acid and 200 mg of Tween 40 emulsifier. Chloroform was completely evaporated. Aliquots of 5.0 ml of this emulsion were transferred into a series of

tubes containing various concentrations of the compounds (50µg/ml). The absorbance was measured immediately (t=0) and after 90 min at 470 nm. The tubes were incubated at 50 0C in a water bath during the test [13].

Superoxide radical scavenging assay

Superoxide anions were generated in a nonenzymatic phenazine methosulfate/ Nicotin adenine dinucleotide system (PMS/NADH) [14]. The reaction mixture contained 1 ml of test solution, 1.9 ml of 0.1 M phosphate buffer (pH 7.4), 1 ml of 20 µM PMS, 156 µM NADH, and 25 µM nitroblue of tetrazolium (NBT) in phosphate buffer (pH- 7.4). After 2 min of incubation at 25°C, the color was read on a spectrophotometer at 560 nm against blank samples that contained no PMS.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured by the ability to scavenge the hydroxyl radicals generated by the Fe^{3}+-ascorbate-EDTA-H_2O_2 system (Fenton reaction) [15]. 1.0 ml contained 100 µl of 2-deoxy2ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 500 µl of the compounds at various concentrations (50-800 µg/ml) in buffer, 200 µl of 1.04 mM EDTA and 200 µM $FeCl_3$ (1:1v/v), 100 µl of 1.0 mM H_2O_2 and 100 µl of 1.0 mM ascorbic acid. Test samples were kept at 37°C for 1 h. One ml of 1 % TBA and 1.0 ml 2.8% TCA were added and were incubated at 100°C for 20 min. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Quercetin was used as a positive control.

Hydrogen peroxide scavenging assay

Hydrogen peroxide solution (2 mM/l) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the compounds (25-400 µg/ml) in distilled water was added to 0.6 ml of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging activity at different concentrations of the compounds was determined and was compared with the standard, α-tocopherol [16].

Inhibition of lipoxygenase enzymatic activity (LOX)

Testing of the effect of the diterpenes on the enzymatic activity of lipoxygenase was tested using the Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. In brief, purified soybean Lipoxygenase was allowed to react with the substrate arachidonic acid in the absence versus presence of test products. The hydroxyperoxides produced as a result of the Lipoxygenase enzymatic reaction were measured in a colorimetric assay, and absorbance read in a microplate reader (BioTek PowerWave, Winooski, VT, USA) at 490-500 nm absorbance.

Protective activity against oxidative damage

Preparation of liver homogenate: The liver was isolated from three normal albino Wistar rats. The organs were weighed and 10% (w/v), the homogenate was prepared in phosphate buffer (0.1 M, pH 7.4 having 0.15 M KCl) using the homogenizer at 4°C. The homogenate was centrifuged at 3000 rpm for 15 min and the clear cell-free supernatant obtained was used for the study.

Preparation of the pro-oxidative solution: The oxidant solution was prepared immediately before its utilization by adding a solution of $FeCl_3$) 100 mM to H_2O_2 0.50% prepared in phosphate buffer (0.1 M, pH 7.4). This solution was used for the investigation of the protective assays on liver enzymes.

Determination of enzymatic antioxidants

Superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GSH**),** and glutathione peroxidase (CSH-Px) activities were measured using commercial kits. All the assay kits were purchased from Cayman Chemical (Michigan, USA), and the procedures were according to the kits instructions. In liver the protein concentration was determined by the Bradford method as described in the Bio-Rad protein assay kit.

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) and the differences between samples were determined by Duncan's Multiple Range test using the Statistical Analysis System programme. P values < 0.05 were regarded as significant.

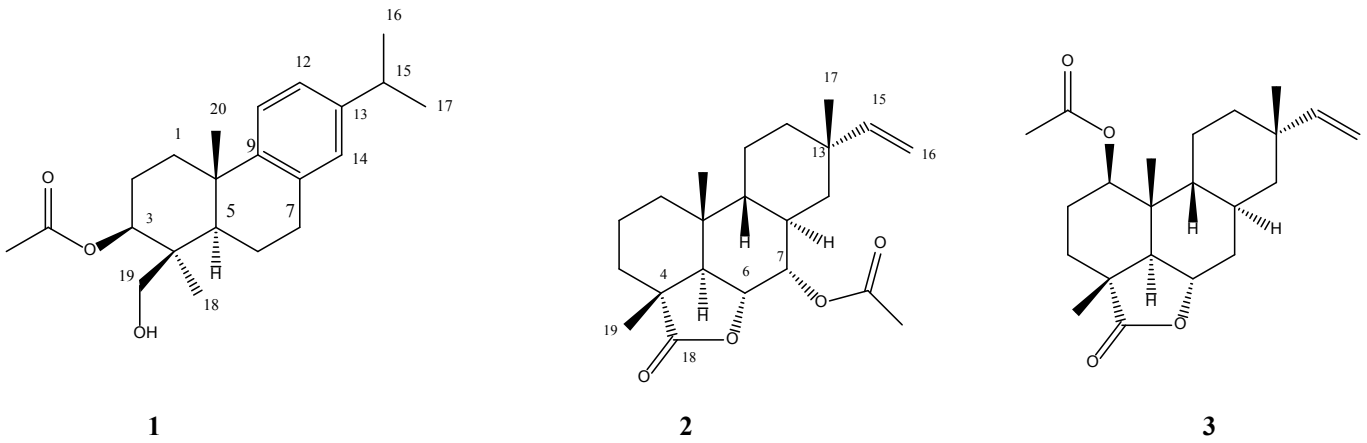
Results

Characterization of diterpenes

Fractionation of residue from hexane extract led to the purification of compound 1, the new identified abietane diterpene was obtained as pale oil. The positive ESI-MS spectrum gave a peak at 344.2372 m/z, corresponding to the molecular formula $C_{22}H_{32}O_3$. The ^{1}H NMR spectra in $CDCl_3$ (Table 1) exhibited twenty two carbon signals suggested that 1 is a diterpenoid derivative with two methyl singlets (δ_H 1.29, s, H_3-18; δ_H 1.25, s, H_3-20), an isopropyl group (δ_H 0.87, d, *J*=7.0 Hz, H3-16; δ_H 0.87, d, J=7.0 Hz, H3-17; δ_H 3.05 sept J=7.0 Hz), an oxygenated methine proton (δ_H 4.61, dd, J=11.5, 5.0 Hz Hα-3) and one oxygenated methylene protons (δ_H 4.07, d, J=11.6 Hz and δ_H 3.56, d, J=11.5 Hz H_2-19). The ^{13}C NMR and DEPT data (Table 1) showed 22 carbon resonances including five methyl, five methylene (one oxygenated), three methine (one oxygenated) and nine quaternary carbons. In addition the signals at δ_H 2.12 (3H, s), δ_C 21.32 and δ_C 167.45 indicated the presence of an acetyl group. These groups accounted for seven degrees of unsaturation deduced from the molecular formula suggesting that 1 had three rings among them a benzene ring. Moreover, analysis of the ^{1}H-^{1}H COSY correlations constructed a fragment –CH_2-CH_2-CHO- (Morikawa et al., 2009). Furthermore, the HMBC correlations from δ_H 4.61 (H-3) to δ_C 167.41 and from both H_3-18 and H_2-19 to δ_C 76.32 (C-3) established an acetoxyl group to be at C-3. The coupling constants of H-3 (dd, J=11.5, 5.0 Hz) and the δ values of H-3 and C-3 were in agreement with those of 3β-acetoxylabieta-8,11,13-triene-12-o1 but markedly different from 3α-hinokiol [17], revealing the acetoxyl group at C-3 to be in β orientation. The presence of an intense NOE correlation between H-19 at δ_H 3.56 (d, J=11.5 Hz) and H_3-20 at δ_H 1.25 in the NOESY spectrum allowed assignment of the oxymethylene (CH_2OH-19) and CH_3-20 groups on the same face of the molecule and in β orientation. Analysis of the ^{1}H-^{1}H COSY and HMQC spectra revealed the presence of four molecular fragments, i.e., an ethylene chain fragment, C-1-C-2; a two-carbon chain fragment, C-11-C-12, comprising aromatic methine carbons, a three-carbon chain fragment, C-5-C-6-C-7, in which C-5 is a methine carbon and C-6 and C-7 are methylene carbons, and a three-carbon chain fragment, C-16-C-15-C-17 which is an isopropyl group (Figure 1). Compound 1 was assumed to have the same configuration as triptobenzene R. Accordingly, compound 1 was determined as abieta-8,11,13-triene-3β-acetyl-19-ol (Figure 2), it was a new diterpenoid named canarien A.

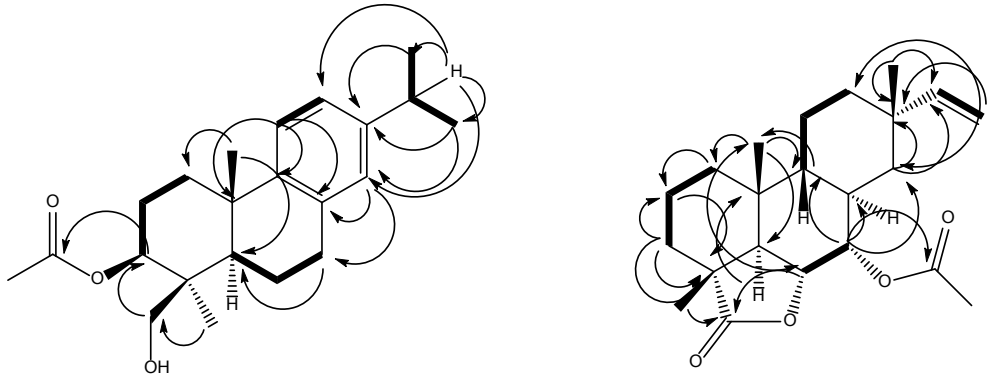
Compound 2 had a molecular formula of $C_{22}H_{32}O_4$ as deduced from a peak at m/z 360.2382 $[M]^+$ (calcd. 360.2301) in the HREIMS. The most significant bands in the IR spectrum appeared at 1728 and 1635 $cm^{-1,}$ can be attributed to a carbonyl and double bond groups. In accordance with the above molecular formula, the ^{13}C NMR spectroscopic (Table 1) and HSQC data showed the presence one ester carbonyl (δ_C 170.33 and 22.01), one γ lactone (δ_C 179.74 and 76.11), and a double bond (δ_C 146.12, and 112.43). The ^1H NMR displayed signals readily recognized for three tertiary methyls as singulets at δ_H 0.83 (H_3-17), 1.23 (H_3-19) and 0.86 (H_3-19). In addition, an ABX system corresponding to a vinyl moiety on a quaternary carbon at δ_H 5.90 (1H, H-15), δ_H 5.11 (1H, H-16 *cis*), δ_H 5.15 (1H, H-16 *trans*), one proton signal for an oxygenated methine at δ_H 4.21 (1H, H-7) were also observed. The combined analysis of the HETCOR, COSY, and HMBC spectra (Figure 2) permitted the assignment for this compound of a Δ^{15}-pimarene skeleton [18], possessing a γ-lactone, and acetyl groups. For the location of the lactone between C-18 and C-6 were the correlations found in the HMBC spectrum between C-18 and Me-19 and between C-6 and H-7 and H-5. The β-orientation of H-6 was suggested by the long vicinal coupling constant (J=11.5 Hz) detected for H-6. The HMBC correlations (Figure 2) of H-6 with C-7 in combination with the ^1H-^1H COSY correlations from H-5 through H-6 to H-7, demonstrated that the acetyl group is attached to the C-7 position. The acetyl was placed on C-7 due to its HMBC correlations with H-5 to C-7, H-6 to C-7 and C-8, H-14 to C-7 and C-9 and H_3-17 to C-14 and the acetyl group should be located on α-orientation because the multiplicity of proton H-7. In the NOESY spectrum, Me-20 correlated with Me-19 and H-6 confirmed the α-disposition of γ lactone ring an orientation that is in agreement with the multiplicity observed for H-6. However, the NOE correlations between H-15 and H-14α and between H-16 *trans* and H-11α and 12α, led to the relative configuration at C-13. The relative stereochemistry of **2** was supported from NOESY experiment. The NOESY correlations observed among signals of H-5 with H-8 indicating that these protons are located on the α-face while the NOESY correlations between H_3-17 and H_3-20 indicated that both methyl groups were on the β-face. Accordingly, the structure of 2 was determined as shown in Figure 1, and this compound has been named canarien B.

The molecular formula of 3, was determined by HREIMS (360.2371, calcd m/z 360.2301). The ^{13}C and ^1H NMR spectra of 3 (Table 1) were similar to those of 2, except for the position of the acetyl group. The planar structture was deduced on the basis of correlation spectroscopy (COSY) and heteronuclear multiple bond connectivity (HMBC) correlations. H-5 exhibited HMBC correlatins to C-1, C-4, C-6, C-7,



1 **2** **3**

Figure 1: Structures of compounds 1-3.



1 **2**

Figure 2: Key HMBC (H—C), COSY (▬) correlation in compounds 1-3.

C-9, C-10, C-18, C-19, and C-20, while H_3-20 showed correlations to C-1, C-5, C-9, and C-10. In addition, A methylene carbon at δ_C 39.51 correlation with δ_H 1.36, 1.27 (CH_2-3), and a methine carbon at δ_C 60.3 with δ_H 1.60 (CH-5). These data established the local structure around the AB-ring juncture. The configuration of one acetyl group (C-1), was addressed on the basis of nuclear Over-hauser effect spectroscopy (NOESY) correlations. The coupling constants of H-1 (dd, *J*=l1.1, 5.0 Hz) indicated the inclusion of a 1,2-diaxial coupling. The α-face orientation of H-1 was confirmed by the NOESY correlations from this proton to H-5 and H_{ax}-3. NOESY correlation between H-5 and H-7 revealed the coplanar relation. Therefore, compound **3** was assigned as canarien C.

Total antioxidant activity

Total phenolic content in hexane extract is very low, only 0.3% so not correlation between total phenolic and the $O_2^{\cdot-}$ inhibitory activity implies that such activity in hexane extract is not based on the presence of polyphenolic antioxidants.

Scavenging activities

The ROS scavenging activities of the three diterpenes, Trolox, BHA, quercetin, mannitol, ascorbic acid, α-tocopherol, BHT and curcumin as reference antioxidant are shown in Table 2. The scavenging of compounds 1-3 on hydrogen peroxide is shown in Table 2 and compared with α-tocopherol, trolox and Trolox butylated hydroxyanisole (BHA) as standards. Compounds 1-3 were capable of scavenging H_2O_2 in a range of 65.05% to 86.54%, producing moderate $^{\cdot}OH$ radical scavenging activity, Addition of the compound 1-3 to the reaction mixture removes hydroxyl radicals and prevents further damage.

The results obtained shown that *P. canariensis* is less efficient HOCl scavenger than standard quercetin (Table 2). Compounds 1-3 presented the lowest scavenging capacity compared to that ascorbic acid (4.17 %). All diterpenes were able to scavenge HOCL, mereover α-tocopherol and Trolox were not able to scavenge HOCL.

ORAC assay has been commonly used to evaluate the total antioxidant capacity of plant foods. In this study, diterpenes and extract were fast and effective scavengers of the ABTS radical and this activity was lower to that of quercetin (Table 2).

Compounds 1-3 and hexane extract were found to be very efficient in scavenging nitric oxide (Table 2). NO scavenging capacity of 1 and extract was similar than the curcumin. The dates on the potency of the inhibition of extract, terpenes 1-3 oxidation by ONOO- are as shown in Table 2. Compound 1 is the most active of the three terpenes with 69.37 % compared with quercetin 72.46%.

Peroxynitrite (ONOO), a cytotoxic reactive species that can be formed by the combination of superoxide (O_2) and nitric oxide (NO) radicals, is generated in vivo.

The β-carotene bleaching test is a convenient test used to measure the ability of a compound or an extract to inhibit lipid peroxidation. This method is based on β-carotene bleaching as a result of reaction between β-carotene and linoleic acid peroxide produced by autoxidation. Results showed that hexane extract from *P. canariensis* and 1-3 exhibited moderate antioxidant activity, with 52.75%, 50.13%, 45.19% and 37.15% respectively of β-carotene remaining after 20 min of assay (Table 2).

The decrease of absorbance at 560 nm with antioxidants thus indicates the onsumption of superoxide anion in the reaction mixture, Table 2 presents the superoxide radical cavenging activity of 100 µg/ml compounds 1-3 and extract in comparison with the same dose of α-tocopherol, compounds 1-3 and extract had a superoxide radical scavenging activity, less than that of references antioxidant at the same concentrations, ($P<0.05$).

The chelating ability of extract and compounds 1-3 was assessed in vitro. The maximum in vitro chelating ability of 1 with a IC_{50} value of 572.26 µg/ml while the minimum IC_{50} value in vitro chelating ability on ferrous ion is 903.30 µg/ml for extract. The extract and compounds 1-3 have chelating ability less than that Trolox with IC_{50} value of 28.63 µg/ml.

The ABTS assay is based on the antioxidant ability to react with ABTS•+ generated in the system. The method is widely used to evaluated antioxidant activity in food and biological systems and a

	Hexane extract	1	2	3	Trolox	BHA	Values standard
•OH scavenging (IC50)	175.17 ± 3.99^a	165.05 ± 5.21^a	179.76 ± 2.19^a	186.54 ± 6.39^a	36.21 ± 1.73^b	28.26 ± 2.65^b	Mannitol 568.51 ± 7.24c
•O_2^- scavenging (IC50)	1727 ± 49.77^c	800.29 ± 44.75^a	1585.35 ± 38.61^c	1905.21 ± 52.03^c	2231 ± 58.87^c	2276 ± 62.34^b	Quercetin 44.61 ± 8.42d
Fe^{2+}-chelating (IC_{50})	903.30 ± 4.83^d	572.26 ± 5.50^d	749.12 ± 5.29^d	844.61 ± 7.20^d	28.63 ± 4.72^b	ND	
DPPH$^{\cdot}$ scavenging (IC50)	22.51 ± 5.45^c	19.81 ± 6.11^c	26.36 ± 3.72^c	28.59 ± 6.30^e	4.65 ± 0.69^b	12.49 ± 1.72^a	Ascorbic acid 5.34 ± 0.19b
ABTS•+ (IC_{50}) µM	441.35 ± 4.56^c	438.30 ± 6.37^c	559.57 ± 8.26^d	587.10 ± 5.39^d	9.73 ± 2.19^b	7.85 ± 1.29^b	
TEAC µM	0.876 ± 0.006^b	1.830 ± 0.023^b	0.621 ± 0.003^c	0.321 ± 0.006^d	ND	ND	
ORAC (1 ORAC unit/1µmol trolox)	37.65 ± 10.23^c	24.55 ± 9.52^a	33.32 ± 7.67^c	36.75 ± 8.59^c	ND	ND	Quercetin 8.56 ± 2.43b
NO$^{\cdot}$ (%)	57.36 ± 3.73^c	60.59 ± 4.12^c	58.38 ± 6.73^c	50.48 ± 7.31^c	ND	ND	Curcumin 59.25 ± 2.90c
ONOO$^{\cdot}$ (%)	65.94 ± 6.29^a	67.37 ± 2.10^a	58.31 ± 4.41^a	52.47 ± 4.28^a	ND	ND	Quercetin 72.46 ± 5.19a
H_2O_2 (%)	150.42 ± 5.80^a	128.13 ± 3.54^c	149.13 ± 5.32^a	167.21 ± 6.58^a	74.41 ± 4.72^b	77.69 ± 8.41^b	α-Tocopherol 67.82 ± 5.34b
SOD scavenging (%)	79.52 ± 5.45^b	73.52 ± 5.45^b	76.28 ± 3.63^b	79.87 ± 4.38^b	44.73 ± 4. 65^a	49.43 ± 3. 18^a	α-Tocopherol 44.63 ± 4.60a
Lipid Peroxidation (%)	52.75 ± 12.32^d	50.13 ± 14.21^d	46.39 ± 9.48^d	37.15 ± 4.88^a	86.50 ± 9.78^b	80.74 ± 6.52^b	α-Tocopherol 69.36 ± 5.14c
HOCl (%)	30.77 ± 3.61^a	33.86 ± 6.04^a	37.25 ± 4.82^b	45.54 ± 5.49^b	ND	ND	Quercetin 27.63 ± 2.57a Ascorbic acid (4.17 ± 0.87c)
FRAP mg/eq Vit C/g DE	9.32 ± 1.03^c	11.56 ± 2.25^c	8.75 ± 2.53^c	7.21 ± 1.53^c	ND	ND	Butylated hydroxyl toluene (BHT) 35.17 ± 5.12 a

IC_{50} value (µg/ml) is defined as the concentration of 50% effect percentage and expressed as Mean ± SD (n = 4). Means values with different superscripts in the same row are significantly different ($p<0.05$), while with same superscripts are not significantly different ($p<0.05$). ND, cannot detected.

Table 2: Antioxidant activities of diterpenes 1-3.

high TEAC value indicated a high level of antioxidant activity [19]. Diterpenes 1-3 showed poor antioxidant capacity in the reaction with ABTS•+, which is expressed as high values of IC_{50} for extract and 1-3 compared to Trolox and BHA (Table 2). TEAC value for for extract and 1-3 were moderate.

The ferric reducing antioxidant power (FRAP) rnethod is based on the reduction of a ferroin analog, the Fe^{3}+ complex of tripyridyltriazine Fe (TPTZ)$^{3+,}$ to the intensely blue colored Fe^{2+} complex Fe (TPTZ)3+ by antioxidants in acidic medium. The FRAP anti-oxidative power of the different tested samples is represented in Table 2. BHT used as standard had the highest and most significant (p>0.05) FRAP antioxidant power.

The scavenging behavior extract and diterpenes 1-3 toward H_2O_2 and OH was examined. This assay shows the abilities of the extract, diterpenes and standard Trolox, BHA and mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in an Fe^{3}+-EDTA-ascorbic acid and H_2O_2 reaction mixture. The results are shown in Table 2. The IC_{50} values of the extract, 1-3 and standard in this assay were 175.17 µg/ml, 165.05 µg/ml, 179.76 µg/ml and 186.54 µg/ml respectivity. The IC_{50} values of the all samples were greater tan than that Trolox (36.21 µg/ml) and BHA (28.26 µg/ml) standard. However, IC_{50} values more efficiently than standard mannitol (571.45 µg/ml). Table 2 did show moderate hydrogen peroxide scavenging activity against H_2O_2, compared with the reference antioxidants followed the order BHA>Trolox>α-tocopherol.

Among the compounds studied 1 showed maximum LOX activity while 2 y 3 show very little inhibitory activity so these results are not presented here. The LOX activity was monitored as an increase in the absorbance at 490 nm, which reflects the formation of hydroperoxylinoleic acid. The highest inhibitory effect was obtained for canarien A of (Table 3).

The SOD activity of the the extract and 1-3 showed the highest activities with values of 5.90 to 6.34 U/mg protein compared to the oxidant control (Table 4). The catalase, glutathione peroxidase and glutathione reductase activities recorded in the different groups is displayed in Table 4. This activity increases with the extract and different compounds tested. This result suggests that enable to act as secondary or preventative antioxidants that retard the rate of oxidation.

Discussion

Fractionation of the hexane extract of the seeds of *Phalaris canariensis* showed the presence of three terpenoids 1-3 which were found to be new compounds and identified as Canarien A-C which were tested for their antioxidants effects. Reactive oxygen species (ROS), which consist of free radicals such as hydroxyl (OH), superoxide (SOD), nitric oxide (NO), radicals and non-free radical's species such as hydrogen peroxide (H_2O_2), singlet (O_2^-), and lipid peroxide (LOOH) are different form of activated oxygen [16]. The most harmful effect of oxygen comes from the formation and activation of ROS which has a tendency to donate oxygen to other substances which results in degradation of cellular components. The hexane extract, 1-3, exhibited moderate strong scavenging effect of hydroxyl radical. Hydroxyl radical is one of the most harmful among the ROS formed in biological systems, causing DNA strand breakage. Hydrogen peroxide, a product of superoxide dismutase reaction was a strong oxidant and it initiates localized oxidative damage leading to disruption of metabolic function and losses of cellular integrity at sites where it accumulate [20]. Excessive levels of H_2O_2 could be minimized through the activities of CAT and different peroxidases. Hexane extract, 1-3 possessed the moderate SOD, CAT, GR and GPX activities. CAT is potential scavengers of H_2O_2, maintains its level and prevented uncontrolled export of this toxic species from organelles to cytosol and competed to remove H_2O_2. Antioxidant enzymes are capable of eliminating ROS and lipid peroxidation products, therefore, they can protect cells and tissues from oxidative damage, antioxidant enzymes include SOD, catalase and peroxidases. SOD is a key enzyme in the natural defense against free radicals, and its deterrninant role has been described. It is well known that the superoxide ion (O2-) is the starting point in the chain production of free radicals. At this early stage, superoxide dismutase inactivates the superoxide ion by transforrning it into hydrogen peroxide (H_2O_2) [21]. These extract and compounds may achieve this activity through several ways including the removal of substrate or singlet oxygen quenching therefore protecting the enzymes. Hydrogen peroxide (H_2O_2), a highly ROS, is produced as the result of SOD reactions. The catalases provide protection against the toxic effects of H_2O_2 that is generated in respiring cells due to its catalytic function.

Superoxide anion is a precursor to the formation in living systems of other ROS such as hydroxyl radical, hydrogen peroxide, or singlet axygen. Therefore, superoxide anion scavenging is important for antioxidant activity and could help prevent oxidative damage. It has detrimental effect on the cellular components in a biological system. It indirectly initiates lipid oxidation by generating singlet oxygen. The production of highly reactive oxygen species such as superoxide anion radicals is catalyzed by free iron through Haber-Weiss reactions [22]

Final concentration (mol/l)	Activity of lipoxygenase	% Inhibition
Control	150.16 ± 13.51	-
Canarien A		
15.10 × 10^{-6}	120.46 ± 6.78	19.77
20.10 × 10^{-6}	25.13 ± 4.32	83.26[a]
25.10 × 10^{-6}	16.21 ± 3.76	89.20 [a]

Results are presented as percent of control ± SD, n = 4, *p*>0.05 vs. control

Table 3: Inhibition of Lipoxygenase enzimatic activity by canarien A.

	SOD activity (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)	GR (U/mg protein)
Control saline	7.43 ± 0.51	151.56 ± 6.21	14.18 ± 1.21	2. 71 ± 0.07
Control oxidant	2.34 ± 0.78	115.61 ± 8.45	7.23 ± 2.11	1.13 ± 0.41
Vit C	5.24 ± 1.48[a] (29)	76.24 ± 5.39[b] (50)	10.23 ± 3.21[b] (28)	1. 90 ± 0.03[b] (30)
Hexane extract	6.0 ± 1.64[b] (19)	88.18 ± 4.98[b] (42)	11.85 ± 2.78[c] (16)	2. 04 ± 0.09[b] (25)
1	5.90 ± 0.98[b] (21)	93.42 ± 3.79[c] (38)	11.01 ± 1.29[b] (22)	2. 15 ± 0.07[b] (21)
2	6.16 ± 0.76[b] (17)	97.24 ± 6.35[c] (36)	11.23 ± 4.19[b] (21)	2. 20 ± 0.04[c] (19)
3	6.34 ± 0.65[b] 15	105.06 ± 4.94[c] (31)	11.61 ± 4.92[b] (18)	2. 24 ± 0.06[c] (17)

Values are expressed as mean ± SD of four replicates. In the same column the values labeled with different letters differ significantly at p>0.05. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR). Percentage.

Table 4: Protective activities of extract and compounds 1-3 against oxidative damage.

which is a reduced form of O_2 has been implicated in the initiating oxidation reactions associated with aging.

The correlation of the two assays ORAC and TEA was not high. A poor correlation may be expected because different free radical sources are used in the two methods. The TEAC assay uses exogenous ABTS radicals, whereas the ORAC assay uses peroxyl radicals. Because peroxyl radicals are the most common radicals found in the human body, ORAC measurements should be more biologically relevant. Both ORAC and TEAC assays are inhibition methods: TEAC reflects the relative ability of hydrogen or electron donating antioxidants to scavenge the ABTS radical cation compared with Trolox while ORAC is a method to measure the scavenging activity of peroxyl radicals. This result suggests that phytochemicals could act as radical scavengers owing to their hydrogen and electron donating capacity and their ability to delocalize/stabilize the resulting radical within the structure.

The reducing capacity may serve as an indicator of their potential antioxidant activity. Higher absorbance indicates a higher reducing power and higher antioxidant activity. The results of the reductive power revealed that the extract and compounds exhibited poor activity. BHT that was used as standard showed the highest activity.

Nitric oxide (NO) is a free radical which plays an important role in the pathogenesis of pain, inflammation. The reaction of NO with superoxide radical generates highly reactive peroxynitrite anion (ONOO-) which is highly toxic for living cell [23].This may be due to the antioxidant properties which compete with oxygen to react with nitric oxide thereby inhibiting the generation of peroxynitrite ($ONOO^-$). Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes [24]. Diterpenoids are responsible for altering the structural and functional behavior of many cellular components. Compound 1 and extract exhibits moderate ONOO- scavenging activity similar to the standard quercetin. However, compounds all show highest NO scavenging effect compared to curcumin standard.

The β-carotene bleaching test is similar to an oil-in-water emulsion system; differences in the solubility's of antioxidant compounds influence their activity in this assay, Hydrophobic antioxidants are reported to perform more efficiently than hydrophilic antioxidants in the β-carotene bleaching test by orienting themselves in the lipid phase and the lipid-water interface, thus directly combating lipid radical formation and β-carotene oxidation. The strong activity of *P. canariensis* components may be due to their higher level of hydrophobic antioxidants. The β-carotene bleaching test is similar to an oil-in-water emulsion system; differences in the solubility's of antioxidant compounds influence their activity in this assay, Hydrophobic antioxidants are reported to perform more efficiently than hydrophilic antioxidants in the β-carotene bleaching test by orienting themselves in the lipid phase and the lipid-water interface, thus directly combating lipid radical formation and β-carotene oxidation [13]. The strong activity of *P. canariensis* components may be due to their higher level hydrophobic of the antioxidants.

Bivalent transition metal ions play an important role as catalysts in oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry [14]. All the test samples showed low chelating effects on ferrous ions compared with Trolox. Chelation property may afford protection against oxidative damage and iron-overload. The two oxidation states of iron, Fe^{2+} and Fe^{3}+ donate or accept electrons through redox reactions that are significant for biological reactions, but they also may be harmful to cells. In excess, iron helps O_2 and hydrogen peroxide to convert into the extremely reactive hydroxyl radical (Haber-Weiss reaction) that cause severe injury to membranes, proteins and DNA [25]. It decomposes lipid hydro-peroxides into peroxyl and alkoxyl radicals responsible for the chain reaction of lipid peroxidation. Chelating ability of plant provides a strategy to avoid free-radical generation and iron-overload by chelation of metal ion.

At sites of inflammation, the oxidation of Cl^- ions by the neutrophil enzyme myeloperoxidase results in the production of harmful ROS, hypochlorous acid [26-34]. HClO has the ability to inactivate the antioxidant enzyme catalase through breakdown of the heme prosthetic group. Catalase inactivation is inhibited in the presence of the extract and 1-3, signifying its HClO scavenging activity compared with ascorbic acid, but lower when compared with the quercetin.

LOX are sensitive to antioxidants, and the most common way of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy-radicals formed in course of enzymic peroxidation [35-37]. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX. These results indicated that the mechanism of the LOX inhibition effect could be partly explained by direct reduction of peroxy- and alkoxy-radicals or may be considered as a superimposition of reactivity of the compounds with free radicals, chelation of Fe^{2+} or partition process in the heterogeneous membrane system [27].

These results agree with those reported for other derivatives abietane and pimarane diterpenes which show significant antipoxidant actrivity [17].

Conclusion

The hexane extract from seeds of *P. canariensis* was found to possess radical scavenging and antioxidant activities. In the present study it is found that the hexane extract of seeds contains abietane and pimarane diterpenes being partly responsible for its marked antioxidant activity as assayed through various models, indicating that they may be useful therapeutic agents for treating radical-related pathological damage and seeds of *P. canariensis* can be used as an accessible source of natural antioxidants with consequent health benefits.

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