Study of *Corylus cornuta* Twig Extracts: Antioxidant, Radical Scavenging, Anti-Enzymatic Activities and Cytotoxicity

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Abstract: In a fist attempt to better understand therapeutic uses of the forest species *Corylus cornuta* by Native People of Eastern Canada, antiradical/antioxidant, anti-enzymatic activities as well as cytotoxicity on Normal Human Keratinocytes (NHK) of *C.cornuta* twig extracts were studied and correlated with their polyphenolic composition. Polyphenolic extracts were obtained by water and ethanolic extractions using two different techniques: maceration and ultrasound-assisted extraction. Antiradical and antioxidant capacities of the extracts were evaluated against DPPH, TEAC, six ROS/RNS and peroxidation lipidic. Anti-enzymatic activities against enzymes involved in oxidation processes were evaluated towards catalase and xanthine oxidase. MTT and Neutral Red assays were used for evaluating the toxicity of the various extracts on NHK after 24 and 48h exposition times. Aqueous extracts were determined to have the highest antioxidant/antiradical capacity against two reactive species involved in inflammatory processes (superoxide anion and nitric oxide) and the lowest toxicity. Their antioxidant/antiradical activities were strongly correlated to their higher content in flavonoids. Ethanolic extracts were determined to have the highest anti-enzymatic activity correlated with their higher content in hydroxycinammic acids and proanthocyanidins. These extracts were also the most toxic, this toxicity correlating with their high level in total phenols. Given that aqueous extracts presented an elevated content in total phenols and flavonoids and showed the lowest toxicity on NHK as well as a high antiradical/antioxidant capacity, they can be considered as the most valuable extracts obtained from *C.cornuta* twigs which is in harmony with traditional uses in which remedies are prepared from twig infusions.

Keywords: *Corylus cornuta*, phenol contents, antioxidant/antiradical, enzymes, lipid peroxidation, cytotoxicity.

1. INTRODUCTION

Beaked Hazel (*Corylus cornuta* Marsh., Betulacea) is the only hazelnut species growing in forest. Several ethnobotanical and ethnopharmacological researches showed that Native Americans used parts of beaked hazel to treat various diseases [1-3]. For example, the Iroquois prepared tea from the branches to treat dental pain and they made necklaces out of pieces of stem to ease tooth pain in young children. The Ojibwas used the root mixed with other species to treat pulmonary hemorrhage. The Algonquins and the Crees drank tea from twigs, branches and leaves to treat heart disease and intestinal disorders. The Potawatomi used the inner bark in medicinal combinations in very much the same way as they used the inner bark of the willow, and also as an astringent and febrifuge [4]. The Abenakis infused the bark with that of American dogwood and willow to treat eye problems. There are numerous testimonies today from people using beaked hazel necklaces or products based on beaked hazel extracts about their positive effects on several pathologies such as arthritis, gastric ulcers, cardiovascular disorders, teeth pains and skin disorders (eczema, psoriasis).

Several studies have already shown the role of oxidative stress in the mechanisms of various human diseases such as cardiovascular [5, 6], lung [7, 8], gastric ulcers [9-12] or even arthritic problems [13, 14]. In normal conditions, our body constantly produces oxidant molecules that are highly reactive oxygen-derived forms the function of which is to provide signaling between cells [15]. The production of primary reactive oxygen species (ROS) such as superoxide anion (O_2^{-}•), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) is regulated by the body by an enzymatic antioxidant system and also by the intake of vitamins related to the daily diet. However, in some cases (UV exposure, stress, alcohol, tobacco, exposure to chemicals), there is an overproduction of ROS. The antioxidant system becomes in that case inefficient and an imbalance is created which is called "oxidative stress". During oxidative stress, the ROS not eliminated by the primary defense system of our body will then oxidize cellular constituents which causes the formation of secondary ROS (such as peroxyl radical ROO•) that are participating oxidation chain reactions which ultimately lead to cell destruction. The oxidative stress is associated with lipid and protein peroxidation, resulting in rapid cell structural damage, tissue injury or gene mutation which ultimately lead to the development of various health disorders such as Alzheimer's disease, cancer, atherosclerosis, diabetes mellitus, and hypertension and finally ageing itself [16]. Under conditions of oxidative stress, excessive

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generation of •NO contributes to the inflammatory response, in part by reacting with O_2^{-}• to form a potent oxidant, peroxynitrite (ONOO–) / peroxynitric acid (ONOOH). Phagocytes naturally produce oxidants such as nitric oxide (•NO), superoxide anion, hydrogen peroxide and hypochlorite ion (OCl^-) as protective agents against cell infection in immune responses [17]. Chronic infections involve a prolonged activity of phagocytes, leading to inflammation and risks associated with it [18]. It is well-known that chronic inflammation is associated with a broad spectrum of neurodegenerative diseases related to aging, such as Alzheimer, Parkinson, amyotrophic lateral sclerosis, age-related macular degeneration, osteoarthritis, rheumatoid arthritis, atherosclerosis, and myocardial infarction [19].

A therapeutic action aimed at correcting oxidant–antioxidant imbalance is a clinical challenge, and has increasingly been the object of research work. Oxidative stress has been found to be controlled by the antioxidant and/or anti-inflammatory effects of dietary polyphenols. Since a variety of oxidants, free radicals and aldehydes are implicated in the pathogenesis of chronic inflammatory diseases, a therapeutic intervention with a variety of polyphenolic antioxidants may therefore be an effective alternative for the treatment of chronic inflammatory diseases [20]. Indeed, the role of polyphenols has been widely demonstrated [21] and there have been new developments in the elucidation of the *in vivo* mechanisms of the health benefits of these compounds [22].

A very few phytochemical or pharmacological studies of *Corylus cornuta* extracts are found in the literature. McCune and Johns [23, 24] demonstrated the high antiradical capacity of *Corylus cornuta* twig and bark extracts, in particular against superoxide and peroxyl radicals. These authors proved that *Corylus cornuta* extracts showed the greatest antiradical activity among 35 medicinal plants used by Indigenous People in the boreal forest of Eastern Canada to treat diabetes symptoms. *Corylus cornuta* were used in particular, to treat symptoms such as diarrhea, heart/chest pain, and sore eyes. However, several researches have been conducted on the European species *Corylus avelanna*. Thus, the tannin fraction of *C. avellanna* bark extracts exhibits antibacterial activity against various strains of bacteria [25]. It can be noticed that most of the phytochemical studies of *C. avellanna* were performed on fruits, leaves, oils or hazelnut kernel and on the by-products (skin, hard shell, green leafy cover, and tree leaf). Indeed, various extracts obtained from hazelnut by-products show antioxidant capacities including the capacity to scavenge DPPH radical and to inhibit lipid peroxidation [26-31]. Analyses of the chemical composition of some hazelnut by-products do indicate the high concentrations of polyphenolic compounds in these extracts [32].

The aim of this study was to better understand the therapeutic uses of *C.cornuta* parts and extracts by Native People of Eastern Canada. For the first time, the composition of *C.cornuta* extracts in terms of various classes of polyphenols was investigated and the correlations between polyphenol content and antiradical/antioxidant, anti-enzymatic activities as well as cytotoxicity were investigated.

2. MATERIALS AND METHODS

2.1. Plant Material

Plant material used in this study was harvested in July 2010, identified and certified by Pure Hazelwood Inc., Sherbrooke, QC. Since 11 years, this company makes necklaces with *C.cornuta* twigs and proposes skin products based on *C. cornuta* crude extracts.

2.2. Extractions

Water and ethanol were used as solvent for extractions. With each solvent, two different extracts were prepared, first by maceration, a classical technique of extraction and the second one using a modern technique of extraction assisted by ultrasounds. Thus, the extracts of *C.cornuta* twigs obtained by maceration in hot water (W_{Mac}), by extraction assisted by ultrasounds in water (W_{UAE}), by maceration in ethanol (Eth_{Mac}) and by extraction assisted by ultrasounds in ethanol (Eth_{UAE}) were analyzed and tested *in vitro*.

2.3. Composition in Polyphenols

2.3.1. Determination of Total Phenol Content

The total phenol (TP) content of the extracts was quantified according to Folin-Ciocalteu's method as described by Scalbert *et al*. [33]. 0.5 mL of sample solution at concentration of 250 µg dry extract/mL was mixed with 2.5 mL of the Folin-Ciocalteu reagent (diluted 10 times by distilled water) and 2.0 mL of an aqueous sodium carbonate solution (75 mg/mL). The final mixture was heated at 50°C during 10 min, after which the absorbance was read at 760 nm against a blank (solution with no extract added). Gallic acid was

used as the standard and TP content was expressed as milligrams of gallic acid (Sigma-Chemical, St. Louis, MO, USA) equivalents (GAE) per gram of dry extract samples (mg GAE/g of dry extract).

2.3.2. Determination of Total Flavonoid Content

The total flavonoid (TFlav) content of crude extracts was determined spectrophotometrically following the method described by Brighente *et al*. [34]. Results are expressed as milligrams of quercetin (Sigma-Chemical, St. Louis, MO, USA) equivalents (QE) per gram of dry extract (mg QE/g of dry extract).

2.3.3. Determination of Total Hydroxycinnamic Acid Content

Total hydroxycinnamic acid (THCA) content was determined as described in European Pharmacopoeia (4^{th} ed, [35]) for *Fraxini folium*. Total hydroxycinnamic acid content was expressed as milligrams of chlorogenic acid (Sigma-Aldrich, St. Louis, MO, USA) equivalents (ChAE) per gram of dry extract (mg ChAE/g of dry extract).

2.3.4. Determination of Proanthocyanidin Content

Anthocyanidin formation in a hydrochloric medium with ferric ammonium sulphate (Laboratoire MAT, Québec, Qc, Canada) as a catalyst was performed as described by Porter *et al*. [36]. The proanthocyanidin content (PAs) was expressed as milligrams of cyanidin chloride (Indofine Chemical Co, Hillsborough, NJ, USA) equivalents (CChE) per gram of dry extract (mg CChE/g of dry extract).

2.4. Trolox Equivalent Antioxidant Capacity

The Trolox Equivalent Antioxidant Capacity (TEAC) of *C.cornuta* crude extracts were evaluated by the method of Prieto *et al*. [37]. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH. A total of 0.3 mL of methanolic sample solution was mixed with 2.7 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The four *C.cornuta* twig extracts were prepared in order to be analyzed at the same concentration in Total Phenols (TP). Extract powders were dissolved in solvent to reach 250 µg in TP expressed in gallic acid equivalent/mL of extract solution. For the blank, 0.3 mL methanol was mixed with 2.7 mL of the reagent. The absorbance of the test sample was measured at 695 nm. Trolox was used as a reference and TAC results were expressed as Trolox equivalents (mg TE/g of dry sample). Extracts TAC were compared to that of the standardized extract Oligopin® prepared at 250 µg/mL.

2.5. Scavenging Capacity Against 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical

Methanolic solutions of 2.10^{-4} M DPPH· and phenolic extract solutions were mixed in the ‘‘Rapid Kinetics Accessory” SFA-11 (HI-TECH Scientific, SALISBURY, England) and the absorbance of DPPH· at 516 nm was monitored. The rate constant of the hydrogen atom transfer pseudo-first order reaction, *k*, was estimated using kinetic equation as described by Diouf *et al*. [38]. Higher rate constant values correspond to higher antioxidant capacities. All extracts were prepared to reach 250 µg in TP expressed in gallic acid equivalent/mL of extract solution and compared to well-known antioxidants: ascorbic acid, BHT, α-tocophérol and propyl gallate (Sigma-Aldrich, Oakville, ON, Canada) used as positive references at the concentration of 250 µg/mL.

2.6. Evaluation of Scavenging Activity Against Reactive Oxygen (ROS)/Nitrogen (RNS) Species

2.6.1. In Vitro Tests, General Remarks

All ROS/RNS scavenging capacities studied in this research were evaluated spectrophotometrically. A commercial pine bark extract, which is a standardized proanthocyanidin-rich extract from French maritime pine bark (PBE), Oligopin® obtained from DRT nutraceuticals (DRT Nutraceutics, Dax, France) and recognized for its several biological activities, was chosen as the positive reference, like in our previous study [39]. Absorbance was measured against a blank solution that contained *C.cornuta* extracts or standard, but without the reagent. A control was performed without adding the *Corylus cornuta* extract or the standard. The percentage of inhibition was calculated by using the following formula:

$$\%\text{Scavenging} = 100^* (A_{control}-(A_{sample}-A_{extract}))/A_{control}$$

where Acontrol is the absorbance of the control (without antioxidant), Asample is the absorbance of the solutions in presence of antioxidant and Aextract is the absorbance of the blank (without reagent). The IC_{50} value, which is the concentration of the sample required to scavenge 50% of reactive species, was determined graphically from the curve plotted for *Corylus cornuta* extracts or standards concentration (µg/mL) vs. % inhibition. For comparison purposes, the

antioxidant efficiency (AE) was defined and plotted as the reciprocal of IC_{50} ($1/IC_{50}$) so that the higher AE value would correspond to better scavenging activity.

2.6.2. Superoxide Radical

Superoxide anion scavenging activity was measured according to the method described by Nishikimi *et al*. [40] with slight modifications. To 1 mL of extract solution (0.01 to 1.0 mg/mL), 1 mL of 0.48 mM β-nicotinamide adenine dinucleotide (NADH) and 1 mL of 0.10 mM Nitro blue tetrazolium (NBT) prepared in 0.01 M phosphate buffer (pH= 7.4) were added. To initiate the reaction, 100 µL of a fresh solution of 60 µM of phenazine methosulphate (PMS) prepared in 0.1 M phosphate buffer was added. After standing for 15 min at 30°C, absorbance was read at 560 nm.

2.6.3.Hydrogen Peroxide

The procedure was adapted from Parij and Nève [41] and performed at 25°C. To 100 µL of extract solution (0.01 to 0.5 mg/mL) were added 2.7 mL of 0.1 M phosphate buffer (pH 7.4), 50 µL of a guaiacol solution at 18 mM and 50 µL of hydrogen peroxide at 500 µM (Sigma-Aldrich, St. Louis, MO, USA). The mixture was allowed to stand during at least 30 min. Then the reaction was initiated by the addition of 100 µL of a fresh horseradish peroxidase solution at 2.10^{-3} mg/mL (~ 1.5×10^{-9} M). Absorbance was read at 436 nm.

2.6.4. Hydroxyl Radical

Hydroxyl radical scavenging activity was measured as described by Smirnoff and Cumbes [42] with slight modification. To 2 mL extract solution (0.01 to 1.0 mg/mL), 600 µL of 8 mM $FeSO_4$ solution, and 500 µL of hydrogen peroxide of 20 mM were mixed. Then, to initiate the reaction, a 3 mM salicylic acid solution was added. The reaction mixture was allowed to stand during 30 min in a bath at 37°C, after which, 900 µL of distilled water were added and the mixture centrifuged during 10 min at 10000 rpm. The supernatant was collected and the absorbance at 510 nm was recorded.

2.6.5. Peroxyl Radical

Peroxyl radical scavenging activity was determined by the method described by López-Alarcón and Lissi [43]. First, 300 µL of the extract solution (0.01 to 1.0 mg/mL) were mixed with 3 mL of a 30 µM of pyrogallol red (PGR, Sigma-Aldrich, St. Louis, MO, USA) solution. Then, 50 µL of a 600 mM AAPH 2,2'-azo-bis-2-amidinopropane hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) solution was added to initiate the reaction. The samples were allowed to stand during 2 h at 37°C. The absorbance was read at 540 nm.

2.6.6. Nitric Oxide

Radical scavenging of nitric oxide (NO) was determined by the method described by Sreejayan and Rao [44] with slight modifications. 200 µL of sodium nitroprusside (SNP, Sigma-Aldrich, Oakville, ON, Canada) (25 mM) was incubated with 800 µL of *C.cornuta* extracts (0.01 to 1 mg/mL) dissolved in phosphate-buffered saline (PBS) (25 mM, pH 7.4) and the tubes were incubated at 37 °C for 2.5 h under normal light exposure. After incubation, the samples were kept in dark at room temperature for 20 min. Thereafter, 300 µl of Griess reagent (Fluka, Ronkonkoma, NY, USA) was added and the absorbance was recorded at 546 nm after 40 min.

2.6.7. Hypochlorous Acid

Hypochlorous acid scavenging activity was determined by the method described by Aruoma and Halliwell [45]. HOCl was produced immediately before use by adjusting NaOCl (1% v/v) to pH 6.2 with dilute H_2SO_4 (25 mM), and its concentration was determined as described by Wasil *et al*. [46]. Each extract or reference solution (0.01 to 1.0 mg/mL) was mixed with 13 mM HOCl (4.3 mM, final concentration) in PBS at pH 7.1 and incubated at 25°C for 10 min, followed by the addition of 16.6 µM bovine liver catalase in PBS. After 15 min incubation at 37°C, the absorbance was read at 404 nm.

2.7. Capacity of the Extracts to Inhibit Lipidic Peroxidation

The capacity of extracts to inhibit lipid peroxidation was evaluated according to the method of Kuo *et al*. [47]. The method consists of measuring the peroxidation of linoleic acid in the presence of oxygen catalyzed by hemoglobin. The results were obtained for hazel twigs extract at phenol content varying from 0.1 to 1 mg / ml. The activity of the extracts was compared to that of four standard antioxidants determined previously: ascorbic acid, BHT, Trolox and propyl gallate.

2.8. Anti-Enzymatic Activities

Due to their antioxidant activity, polyphenolic compounds, by donating electrons, could change the redox state of enzymes, particularly those that contain metals as their prosthetic groups or react with free radicals generated at the active site of the enzyme,

thereby affecting enzyme activities [48]. The effect of *C.cornuta* twig extracts on two enzymes that 1) catalyze the oxidation of their substrate by molecular oxygen and hence generate either intermediate or end product-reactive oxygen species 2) limit oxidation process by scavenging reactive oxygen species was investigated. Two enzymes involved in antioxidant processes were examined: one required to combat reactive oxygen species that cause damage to cells (catalase) and the other stimulating the production of ROS (xanthine oxidase)

2.8.1. Xanthine Oxidase

The capacity of the extracts to inhibit xanthine oxidase enzyme was determined by method described by Cos *et al*. [49].The formation of uric acid is measured spectrophotometrically at 290 nm. The xanthine oxidase (Sigma Aldrich, EC 1.17.3.2.) catalyses the formation of uric acid and superoxide radical towards the reaction between xanthine and oxygen. The extracts containing total phenols concentrations ranging between 0.01 and 1 mg/mL were tested. The activity of the extracts were compared to those of the Oligopin®, quercetin and curcumin (Sigma Aldrich).

2.8.2. Catalase

The effect of the extracts on the catalase (Sigma Aldrich, EC 1.11.1.6) an anti-oxidant enzyme was deduced and discussed from the results of the *in vitro* test performed with HOCl (*2.6.7*). Finding out that certain extracts, beyond certain concentrations, had tendency to favour catalase degradation instead to inhibit it, our aim was to quantify the dose beyond which the extracts started to have an antagonist effect against this antioxidant enzyme, becoming "pro-oxidant". The bovine liver catalase was used in this study.

2.9. Toxicological Evaluation of *Corylus cornuta* Extracts on Normal Human Kératinocytes

2.9.1. General Remarks

Extracts dissolved in culture medium were filtered on 0.22 µm Millex GV filters (Millipore, Nepean, Canada) and exposed to keratinocytes during 24 and 48 hours. The main outcome criterion used for evaluating the toxicity of extracts was the determination of toxicity value (IC_{50}). IC_{50} represents the minimal toxic dose required to cause the decrease of cell viability by 50 % compared with the untreated cells, which were set to represent 100 % of viability. This value was estimated from a dose-response curve, using a non-linear regression algorithm [50]. Lower IC_{50} values indicated higher toxicity.

2.9.2. Cell Culture

Normal human keratinocytes obtained from a breast reduction surgery at passage 1 were cultured (6,7 x 103 cells/cm^2) in the presence of a lethally irradiated 3T3 feeder layer (2,0 x 104 cells/cm^2) in a combination of Dulbecco-Vogt modification of Eagle's medium (DMEM) with Ham's F12 (3:1) supplemented with 5% Fetal Clone II serum (Hyclone, Scarborough, Ontario, Canada), 5 µg/mL insulin (Sigma-Aldrich, Oakville, ON, Canada), 0.4 µg/mL hydrocortisone (Cedarlane, Hornby, Ontario, Canada), 10^{-10} M cholera toxin (ICN Biochemical, Montréal, Québec, Canada), 10 ng/mL human epidermal growth factor (EGF) (Austral Biological, San Ramon, CA), 100 UI/mL penicillin (Sigma) and 25 µg/mL gentamicin (Schering, Pointe-Claire, QC, Canada). All cultures were incubated at 37 °C in an 8% CO_2 air atmosphere and changed three times a week with the media previously described. At 80 % confluence, keratinocytes were subsequently subcultured after differential dissociation of the feeder layers with 0.05 % trypsin-0.01 % ethylenediamine tetraacetic acid (EDTA) treatment for 5 min. Cells at passage 3 were used in this study.

2.9.3. Neutral Red Test

The cytotoxicity neutral red test is based on the ability of live cells to uptake and bind neutral red (*NR*). NR is a positively charged dye that easily diffuses through the cellular membrane of the cells, accumulates in the cellular cytoplasma and is stored in the acidic environment of lysosomes. The principle of the test is based on the fact that NR can only be adsorbed by and bound to live cells, while this ability declines in damaged or dead cells. The amount of accumulated NR is thus directly proportional to the amount of live cells in the cell culture. It is one of the most used cytotoxicity tests with many biomedical and environmental applications. The study was performed on three cultures of normal epithelial keratinocytes of adult humans (NHEK). Concentrations (1000, 500, 250, 125, 62.5, 31.25 and 15.625 mg / mL) of each product were tested replicas (6). The confluence of NHEK at the time of exposure was between 30 and 50%. After a 48 hours exposure, viability was measured by using the neutral red (Neutral Red). A positive control (sodium lauryl sulfate, SDS) was included in each experiment. The results are expressed as IC_{50}, which corresponds to the dose of the product at which there is 50% cell

viability. The IC_{50} was calculated from a nonlinear equation (Hill model) using the software GraphPad Prism version 5.00.

This test was performed on cell cultures. In most applications, the cells are placed in culture plates with 96 wells 6.4 mm in diameter. Each well can only be used for a single determination. This experimental device allows testing of multiple chemical concentrations of samples in duplicate, with positive and negative controls. After the treatment of cells with chemical products of concentrations ranging between at least two orders of magnitude (for example, between 0,01 mM and 1 mM) and by chemical products representing positive and negative control, the cells are rinsed and treated with Neutral Red dye. Only the viable cells are capable to incorporate and bind the dye. The dye can either be added immediately after the elimination of the tested chemical product, thus determining its immediate effect, or it can be added at variable intervals after the product elimination, in order to determine its cumulative or deferred effect. The colour intensity corresponds to number of viable cells in each well. To measure this a spectrophotometer equipped with plate reader programmed to measure the intensity of each of 96 wells of culture plate is used. This automated method yields quickly the results of the concentration-response study and of the statistical analysis.

2.9.4. Test with MTT

Another toxicity test is the relatively simple method with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) which is reduced to blue formazan in living cells. The MTT is a tetrazolim type of dye which is reduced by mitochondrial enzymes of the viable cells only. Therefore, the color intensity measured is directly proportional to the degree of integrity of mitochondria. This test is convenient for detection of cytotoxic compounds in general but also as the test for detection of agents attacking specifically the mitochondria. Normal human keratinocytes were plated into 96-well plates at 1.1 x 10^4 cells/cm^2 with 2.0 x 10^4 cells/cm^2 of 3T3 feeders for 3 days until 70 % confluent. Another series of feeder cells culture also seeded at 2,0 x 10^4 cells/cm^2 were used to further evaluate the contribution of irradiated cells to the formation of formazan . After 24 and 48 hours of exposure to the extracts and culture media, cells were washed with phosphate buffered saline (PBS) and the solution of methyl tetrazolium MTT (Sigma) (1mg/ml) was added to each well during 2 hours. Then, the culture medium (0.2 ml) was aspired and replaced with an equal volume of acidic Isopropanol (EMD Chemicals) to dissolve the intracellular dark-blue formazan crystal formed. After a few minutes at room temperature, absorbance was read at 570 nm. The cytotoxicity to keratinocytes was then determined by subtracting the cell viability measured in keratinocyte + feeder culture with that in feeder-alone culture as described by Poon and Burd [51].

2.10. Statistical Analysis

The results are expressed as averages of three measurements ± standard deviation. The ANOVA variance analysis was applied to compare the averages by Duncan test. The statistical significance threshold was set at $p < 0.05$. The data were collected using software R-Gui 2.112 http://www.R-project.org. The non-parametric Spearman's correlation test was used for correlations ($p<0.05$).

3. RESULTS AND DISCUSSION

3.1. Extraction Yields and Polyphenolic Composition

The extraction yields and the chemical composition of crude extracts from *Corylus cornuta* twigs are presented in Table **1**. The results show that water extraction assisted by ultrasounds (W_{UAE}) is less effective than other extraction types in terms of extraction yield. The extraction by maceration in hot water is, on the contrary, the most effective in terms of extraction yield. It is well-known that extraction with hot water is a good method to recover polyphenols due to their hydrophilic functions and a higher mass transfer at high water temperatures [33]. Unlike aqueous extractions, the ethanol, extraction yields are similar regardless of the technique In the case of ethanolic extractions; ultrasounds permit to reach the maximum yield in a shorter time. Indeed, in preliminary experiments, optimization of ultrasonic extractions was done by following total phenol content (TP) in extract solutions (ethanol or water) prepared with the same initial mass of *C. cornuta* twig dry powder, the same ratio of solid/liquid and in a temperature controlled environment (data not shown). Lavoie *et al*. [52] showed that optimization of extraction assisted by ultrasounds can lead to the recovering of a great amount of extractives in a shorter time and also can permit to select bioactive compounds. Ultrasound is known to facilitate the extraction of low molecular weight molecules. It was reported that ultrasound promotes the extraction of bioactive polyphenols [53].

Table 1: Extraction Yields, Total Phenol, Total Flavonoids, Total Hydroxycinnamic Acids and Proanthocyanidins Contents Determining Chemical Composition of *Corylus cornuta* Twig Extracts Obtained by Water and Ethanolic Extractions

*E*xtracts	Yields* (%)	TP (mgGAE/ g dry extract)	Total Flav		Total CinnAc		PAs content	
			(mg QE / g dry extract)	% **flav	(mg ChAE/ g dry extract)	%** CinnAc	(mg CChE/ g dry extract)	%**PAs
W_{MAC}	10.29	325.0 ± 9.1^{d}	15.2 ± 0.6^{a}	4.7 ± 0.5	8.7 ± 0.7^{d}	2.7± 0.2	10.5 ± 0.6^{e}	3.2 ± 0.2
W_{UAE}	7.69	174.9 ± 1.1^{e}	7.2 ± 1.2^{b}	4.1 ± 3.8	12.6 ± 0.4^{d}	7.2 ± 0.2	15.1± 0.6^{c}	4.7 ± 0.2
Eth_{MAC}	9.84	410.7 ±14.5^{c}	2.1 ± 0.2^{d}	0.5 ± 0.1	44.6 ± 4.0^{b}	10.9 ± 1.0	21.5 ± 0.2^{b}	6.6 ± 0.1
Eth_{UAE}	9.96	433.9 ± 7.2^{b}	4.3 ± 0.6^{c}	1.0 ± 0.3	18.8 ± 2.6^{c}	4.3 ± 0.6	12. 4 ± 0.3^{d}	3.8 ± 0.1
Oligopin®	-	572.9 ±12.1^{a}	7.4 ± 0.1^{b}	-	335.5 ± 3.3^{a}	-	105.0 ± 9.9^{a}	-

Means with different letters in the same column are significantly different at p<0.05 (ANOVA, followed by Duncan test). TP = total phenols content; Total Flav = total flavonoid content; Total CinnAc = total hydroxycinnamic acids content, PAs content = proanthocyanidin content. W_{MAC} = extract obtained by maceration in hot water; W_{UAE} = extract obtained by extraction assisted by ultrasounds in water; Eth_{MAC} = extract obtained by maceration in ethanol (at ambient temperature); Eth_{UAE} = extract obtained extraction assisted by ultrasounds in ethanol.
* % (dry wt plant material) ** % correspond to the proportion of the polyphenol class reported to the total Phenol content of the considered extract.

Thus, depending on the solvent used, ultrasonic extraction appears as a good alternative extraction technique compared to conventional methods because of its high efficacy, lower energy consumption and lower water consumption (no need for refrigeration because no reflux). The extraction time remains the most important parameter that marks the difference between the conventional techniques and ultrasound-assisted technique [54]. The effectiveness of this technique has also been demonstrated in several recent studies [55, 56].

Results in Table **1** confirm the preliminary observations. Indeed, TP in mg gallic acid equivalents / g of dry extract was significantly higher ($p < 0.05$) in ethanolic extracts than in aqueous extracts. Moreover, Eth_{UAE} extract contains a significantly higher TP than Eth_{MAC} extract. Compared to the standardized extract Oligopin® which is purified to be enriched in polyphenols, *C.cornuta* ethanolic extracts had lower TP contents but considering that these are crude extracts, they can be regarded as rich in polyphenols. On the other hand, the lowest content in TP was determined in W_{UAE} extract composed of only 17% of phenolic compounds which is significantly lower than W_{MAC} extract. It is clear from this analysis that the extraction technique and the solvent have a direct influence on TP content.

In general, the aqueous extracts contain more flavonoids than ethanolic extracts while ethanolic extracts are richer in hydroxycinnamic acids (Table **1**). TheW_{MAC} extract has a double Total flav content compared to the standardized extract, Oligopin®, while the total phenols content of W_{UAE} is comparable to Oligopin. It is interesting to observe that even if W_{UAE} extract has the lowest TP content, the flavonoids represent 4.1 ± 3.8 % of polyphenols present in this extract, which is similar to W_{MAC} extract (4.7 ± 0.5 % of its TP content) and significantly higher than Eth_{MAC} extract (0.5 ± 0.1% of its TP content). Ultrasounds seem therefore to favor the extraction of this class of polyphenols. Indeed, Total flav content in Eth_{UAE} extract is double of Eth_{MAC} extract. Phenolic acids contents in *C. cornuta* dry crude extracts are much lower than that determined for Oligopin®. In the same way, levels of proanthocyanidins in beaked hazel twig extracts are ten times lower than that of Oligopin®. Eth_{MAC} extract is the one which was determined to have the highest contents in these two classes of polyphenols. One should keep in mind however, that other classes of polyphenolic compounds which are not taken into account in this analysis, may play a key role in bioactivities of these extracts.

3.2. Trolox Equivalent Antioxidant Capacity

Figure **1** shows that all beaked hazel twig extracts prepared at the same concentration in TP in the extract solution demonstrated a TEAC greater than or similar to Oligopin®. The highest TEAC was obtained with W_{UAE} extract, meaning that 1 mg of W_{UAE} extract TP has a reducing power equivalent to about 14 mg of the pure antioxidant, Trolox. Thus, for an equivalent concentration in TP in µg/ mL of extract solutions, *C. Cornuta* extracts show quasi identical antioxidant capacity.

3.3. Radical Scavenging Capacity Against DPPH

The results presented at Figure **2** show that *C.cornuta* twig extracts present a high capacity to

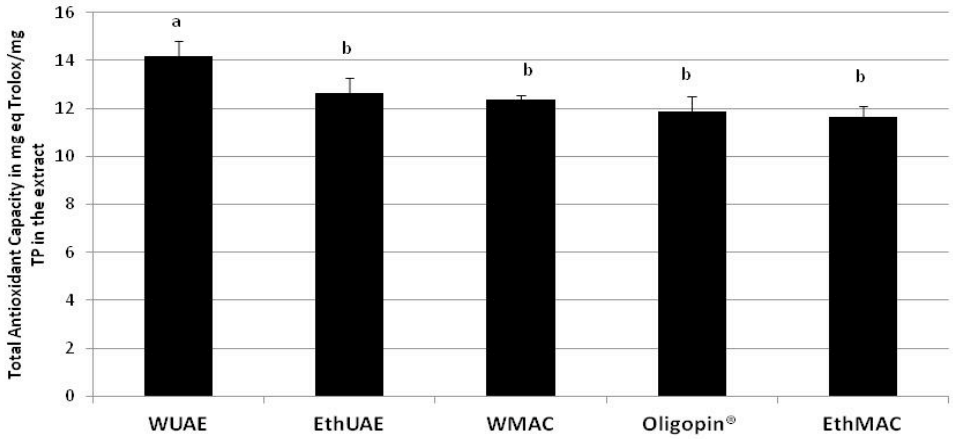

Figure 1: Total Antioxidant Capacity (TAC) of *C.cornuta* twig extracts taken at the same concentrations in total phenols (250 µg of TP in GAE/mL of extract solution) and compared to Oligopin® at 250 µg/mL.
Bars with different superscript letters are significantly different at p <0.05 (ANOVA followed by Duncan's test). The extract with the letter "a" is the most antioxidant.

scavenge the synthetic radical, DPPH. Indeed, compared to the standard antioxidants (α-tocopherol, propyl gallate, ascorbic acid and BHT), commonly used in food or cosmetics, all extracts exhibit a comparable or better antiradical activity. The pure antioxidant propyl gallate is the one which provides the strongest antiradical activity against DPPH. Among *C.cornuta* twig extracts, the highest rate constant was determined for W_{MAC} extract and thus the W_{MAC} extract has a capacity to react quickly and scavenge DPPH radical. This extract has an activity comparable to ascorbic acid (vitamin C).

3.4. Evaluation of Scavenging Activity Against Reactive Oxygen and Nitrogen Species (ROS/RNS)

Results presented in Table **2** put in evidence the chemical antiradical properties of *C. cornuta* extracts. However, one should keep in mind that these results cannot be used to interpret the complexity of the interactions of antioxidants *in vivo.* While there is no ambiguity about the validity of the *in vitro* data and these can be used as basis for planning *in vivo* and clinical studies, an *in vitro* assay cannot be extrapolated to an *in vivo* situation. Therefore, the

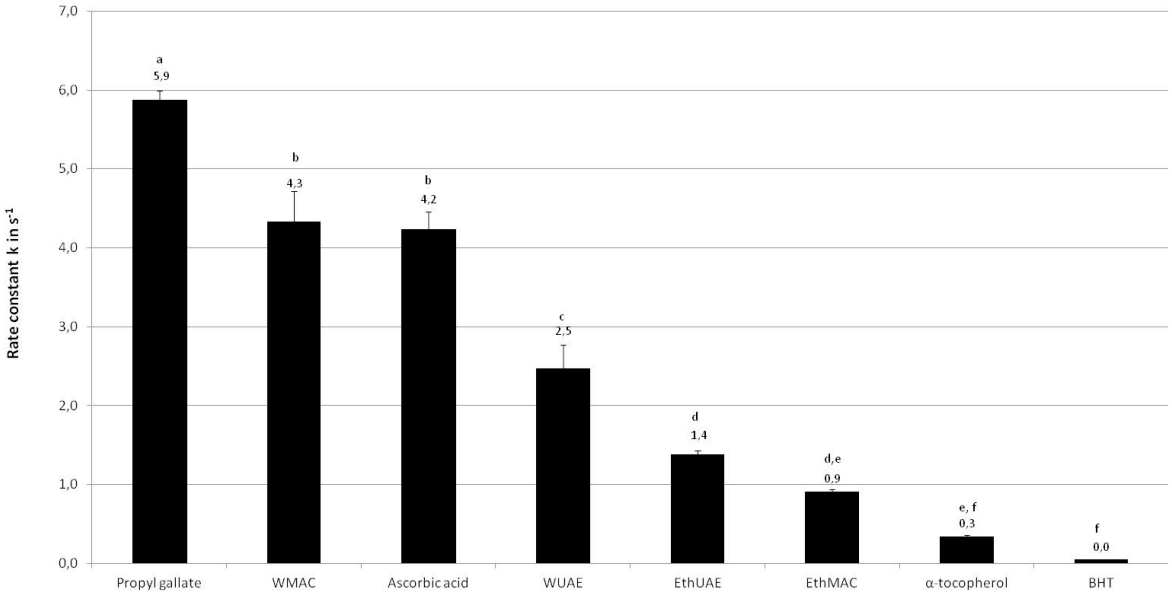

Figure 2: Constant rate (k in s-1) of DPPH scavenging by *C. cornuta* twig extracts at the concentration of 250 µg in Total Phenols/mL of extract solution. Extract activities were compared to standard antioxidant at the concentration of 250 µg/mL.
Bars with different superscript letters are significantly different at p <0.05 (ANOVA followed by Duncan's test). The extract with the letter "a" is the most antioxidant.

Table 2: IC_{50} Values Determined for *C. cornuta* Extracts Scavenging Various ROS and RNS

ROS	PRIMARY ROS			SECONDARY ROS/RNS		
IC_{50} *	OH·	H_2O_2	O_2^{-}·	ROO·	NO·	HOCl
W_{MAC}	850.5 ± 52.0[a]	223.8 ± 20.3[c]	6.3±1.5[a]	333.2±7.5[b]	66.6±5.5[a,b]	n.d
W_{UAE}	828.0 ± 33.5[a]	128 ± 16.8[a]	11.5±1.5[a]	249.3±11.2[a]	48.9±6.1[a,b]	n.d
Eth_{MAC}	inactive	181.1 ± 20.2[a,b]	562.5±133.6[c]	377.4±25.0[b]	87.9±21.9[a,b,c]	inactive
Eth_{UAE}	inactive	226.7 ± 33.4[c]	70.7±44.6[a]	516.6±41.6[c]	144.3±31.5[a,b,c]	inactive
Oligopin®	1034.6 ± 20.9[b]	347.9 ± 88.3[d]	119.1±9.3[a]	1072.8±51.6[d]	2083.3±196.3[d]	471.8±27.5[b]
Curcumin	-	-	492.7±109.4[c]	-	16.3±4.7[a]	
Quercetin	-	-	295.4±8.9[b]	-	-	-
Ascorbic acid	-	-	-	-	-	864.3±19.1[c]

Means with different letters in the same column are significantly different at $p<0.05$ (ANOVA, followed by Duncan test). *IC_{50} are expressed in µg TP in GAE eq/mL of extract solution instead of the conventional µg dry extract/mL solution.

supporting evidence of *in vivo* antioxidant capacity should also be provided. These assays do not take into account the bioavailability, *in vivo* stability, *in vivo* storage patterns by tissues and the reactivity of the sample matrix *in vivo*, nor do they reflect the well documented synergistic effects of antioxidants.

IC_{50} presented in Table **2** demonstrate that aqueous extracts are the most effective extracts to scavenge OH·, O_2^{-}·, ROO· and NO· ($p< 0.05$). W_{MAC} extract is the most active of *C. cornuta* extracts with a capacity to inhibit 50% of the superoxide radical at a very low concentration. The analysis of dose-response curves showed also that activity of the extracts increases in a dose-dependence manner. At a maximum dose tested of 500 µg of TP / mL of extract solution, aqueous extracts exhibited the highest percentage of inhibition (≈ 90%) of superoxide anion which is statistically comparable to the Oligopin® taken at the same concentration. When the extracts were exposed to H_2O_2, they were determined to be significantly more active than Oligopin®, W_{UAE} extract being the most active when considering its low IC_{50} in TP / mL of extract solution ($p< 0.05$). But it should be taken into account that it corresponds to 730 µg / mL of dry crude extract. In fact, in terms of crude extract Eth_{MAC} would be more effective because it has a higher TP content per g of dry extract (Table **1**) and would require a lower dose (≈ 440 µg / mL) of crude extract to reach a level of phenols permitting the inhibition of 50% of H_2O_2. *C. cornuta* twig extracts were weakly active against OH•. The two aqueous extracts have lower IC_{50} than the ethanolic extracts and Oligopin® but the dose required in µg TP/mL of extract solution to inhibit 50% of the hydroxyl radical OH • is over 500 µg TP /mL of extract solution, which would correspond to more than 2000 µg of dry extract/mL. Hydroxyl radical is produced by the reaction of superoxide radical with hydrogen peroxide. We have shown that *C.cornuta* aqueous extracts strongly inhibit superoxide radical and hydrogen peroxide (Table **2**). So despite the fact that these extracts are only slightly active against the hydroxyl radical itself, they are capable of strongly inhibiting its formation *via* their high reactivity with both primary ROS which are involved in hydroxyl radical formation. When exposed to peroxyl radical, W_{UAE} extract is the most active but the other extracts are also effective and all extracts exhibit an IC_{50} lower than that of Oligopin® (p <0.05). Here again, the relative proportions of the various classes of polyphenols present in W_{UAE} extract seem to play a role in the activity of this extract compared to its analogue obtained by maceration. The study of extract capacity to scavenge NO· demonstrates that the lowest IC_{50} is obtained with the reference, curcumin, a well-known anti-inflammatory compound [44]. However, the aqueous extracts of *C. cornuta* twigs present a comparable activity to that of this pure compound (p<0.05). Besides, all extracts are significantly more active than Oligopin® which has an IC_{50}> 2000 µg/mL and which can be considered as inactive against NO•. The two most active extracts against NO• demonstrate the highest proportion in flavonoids in their phenolic composition. Test of the activity of *C. cornuta* twig extracts against the powerful oxidant HOCl released during inflammatory processes was performed *in vitro* in the presence of natural antioxidant enzyme system, catalase, an enzyme that destroys the primary ROS, H_2O_2 to produce water and oxygen. Indeed, HOCl is an oxidant enough powerful to disable this antioxidant enzyme. The capacity of the extracts to inhibit the deactivation of the enzyme and so, to scavenge HOCl was evaluated at several concentrations in µg TP/ mL of the extract solution. The results presented in Table **2** show that *C. cornuta* twig

extracts were not active or showed very weak capacity to scavenge HOCl compared to ascorbic acid or Oligopin®. Activity was only detected for the two aqueous extracts which demonstrate inhibition of the catalase deactivation by HOCl inferior to 50%. W_{MAC} extract is active in the range of 176.3 ± 15.4 to 863.3 ± 14.8 µg total phenols/ mL of extract solution and W_{UAE} extract is active in the range of 131.4 ± 12.9 to 668.8 ± 16.7 µg total phenols/ mL of extract solution. In these ranges of concentrations, both extracts inhibit around 35% of catalase deactivation by the oxidant HOCl.

Overall, the results obtained indicate that polyphenols contained in W_{UAE} extract are the most efficient radical scavengers, whereas ethanolic polyphenolic extracts are quite similar in their behavior against radical species. The interpretation of the activities either based on the concentration in total phenols (such as purified extracts) or on the concentration in crude extract (containing a mixture of various kinds of molecules in addition to phenols) allowed us to identify the ultrasonic water extract as the one with the broadest spectrum of different classes of phenols in proportions (% w/w of each class of phenols/ total phenol content). Thus a purification of this extract could provide an extract with even greater activity.

3.5. Capacity of the Extracts to Inhibit Lipidic Peroxidation

All lipids containing unsaturated fatty acids regardless of their origin (vegetable oils, fish oils, animal fats, cell membranes, lipoproteins) are susceptible to peroxidation. The study of the mechanisms of lipid peroxidation and its prevention by antioxidants is experiencing a resurgence of interest due to the implications of this phenomenon in the areas of nutrition and health. In food, for both humans and animals, lipid oxidation is a serious problem for the food industry that uses more highly unsaturated fatty acids extremely susceptible to oxidation. The oxidation of dietary fat leads to qualitative (rancidity, odor) and nutritional (loss of vitamins and amino acids) alterations or even toxicity caused by products of lipid peroxidation (accumulation of peroxides, aldehydes, etc. in food) [57]. This remains true for cosmetics, themselves rich in polyunsaturated fatty acids. Peroxidation has been related to cellular aging and various diseases such as Parkinson's and Alzheimer's as well as schizophrenia, atherosclerosis, inflammatory diseases, heart damage and reperfusion ischemia [58]. The study of the lipid peroxidation inhibition of linoleic acid (an Omega-3) permits to evaluate the extracts in a system closer to reality. This test evaluates the influence of extracts on the oxidative stability of lipids present in our bodies as well as in manufactured products such as food (for humans and animals) and cosmetics rich in polyunsaturated fatty acids.

The activity of the extracts was compared to that of four standards used as antioxidants: ascorbic acid, BHT, Trolox and propyl gallate. The results presented in Table **3** show that W_{MAC} extract with an IC_{50} of 30.7 ± 53.0 µg / mL total phenols (corresponding to a mass of crude extract of 94 µg/mL) has an activity significantly higher than that of the other extracts and all tested antioxidant references (p<0.05). W_{UAE} is the second most powerful extract, which inhibits 50% of lipid peroxidation at a very low concentration. Its activity is comparable to that of BHT. EthMac extract which presents an activity comparable to that of vitamin C is also more active than the ultrasonic extract obtained with the same solvent. The EthUAE extract is the least efficient and it is less active than all antioxidant references.

Table 3: IC_{50} of *C. cornuta* Twig Extracts Determined to Evaluate their Capacity to Inhibit Lipidic Peroxidation

Extracts	IC_{50} (µg of total phenols/mL of extract solution)
W_{MAC}	30.7 ± 53.0^{a}
W_{UAE}	$96.7 \pm 36.7^{a,b}$
Eth_{MAC}	$224.7 \pm 81.3^{b,c}$
Eth_{UAE}	549.5 ± 36.0^{e}
Ascorbic acid	244.7 ± 24.8^{c}
BHT	$129.8 \pm 15.9^{a,b,c}$
Propyl gallate	255.7 ± 18.6^{c}
Trolox	457.3 ± 57.3^{d}

Means with different letters in the same column are significantly different at p<0.05 (ANOVA, followed by Duncan test).

3.6. Correlations Between Antioxidant/Antiradical Activities and Polyphenolic Composition

Spearman's correlation data presented in Table **6** show that the correlation coefficients characterizing the relationship between antioxidant / antiradical efficiencies (calculated as AE = $1/IC_{50}$) measured for each *in vitro* test and the content of total phenols (TP) determined in aqueous and ethanolic extracts of *C.cornuta* twig extracts, are all significantly (p-value <0.05) negative, except for TEAC for which no

significant correlation can be observed. Thus, in general, more *C. cornuta* extracts present a high TP content per gram less they are antioxidant/antiradical. Several studies have been made concerning the relationship between the phenolic structure and anti-oxidant activity [59], but no relationship has been elucidated because of the many different evaluation systems used to test for anti-oxidant activity. Several studies have demonstrated a linear relationship between anti-oxidant capacity and total phenolics [60, 61]. According to them different phenolic compounds have different responses in the Folin-Ciocalteau method. Negative correlation between TEAC and total phenolic content was found in strawberries [62]. The authors supposed that this phenomenon could be due to a high content in ascorbic acid. Kahkonen *et al*. [63] found that no significant correlations could be found between the total phenolic content and antioxidant activity of various plant extracts and considered that the antioxidant activity of an extract cannot be predicted on the basis of its total phenolic content. Correlation coefficients between hydroxycinnamic acids (THCA) and antiradical activities were not significant, except for DPPH scavenging, for which the coefficient value is significantly negative (p-value <0.05). This class of polyphenolic compounds seems not to be involved in the activity of *C. corylus* twig extracts against reactive species or lipidic peroxidation. On the other hand, the results presented in Table **6** indicate clearly the strong influence of flavonoids on the general antioxidant/antiradical activities of *C.cornuta* twig extracts, including their capacity to inhibit lipid peroxidation. The only ROS not affected by flavonoids is HOCl. The various activities are directly proportional to flavonoid content of the extracts and correlate significantly (p-value <0.05) with them, except for nitric oxide for which the correlation is less significant (p-value <0.1). The most significant correlation has been found between Total flavonoid content and superoxide anion scavenging. In the literature, it is well-established that flavonoids are excellent scavengers of superoxide anion O_2-• [64]. Regarding the correlations between proanthocyanidin content (PAs) and antioxidant / antiradical activities, it was found that the capacity of the extracts to scavenge superoxide anion and DPPH radical is negatively correlated with PAs (p-value <0.05) while a low positive correlation is found between PAs and nitric oxide scavenging.

As the results of the study of the correlations between the phenolic composition and the antioxidant/antiradical activities of *C.cornuta* twig extracts indicate, the strong influence of flavonoids has been determined, while there are no significant effects of hydroxycinnamic acids or proanthocyanidins.

3.7. Enzyme Modulators

From the general point of view, the ethanolic extracts are those which demonstrate the highest capacity to react with enzymes. The first observation in this sense can be made from analysis of the results obtained by *in vitro* test performed to determine antioxidant activity of *C. cornuta* twig extracts against HOCl in presence of catalase (Figure **3**). Catalase is an antioxidant enzyme that is capable of scavenging oxygen and organic free radicals such as H_2O_2. It was interesting to see *via* this test that the two ethanolic extracts were acting by "promoting" catalase deactivation from the very low dose in total phenols (<50 µg TP/ mL extract solution). This phenomenon appears at the dose-response curve by a negative inhibition rate (Figure **3**). This behavior could be explained by complex combination of interactions and also by solubility restrictions as observed by Aruoma *et al*. [45]. Mathematic models applied on the dose-response curves permitted us to estimate for each extract the concentration from which a deactivation of the catalase is observed in presence of these extracts (Table **4**). Results show that aqueous extract demonstrate this antagonist effect only at very high dose in TP in the medium. It has already been proved that high dose of polyphenolic antioxidants can provoke pro-oxidative mechanisms [65, 66]. Moreover, it is known that bovine catalase used for this test is easily deactivated [67]. Like traditional heterogeneous catalysts, enzymes also suffer from the common failing of being rather easily and irreversibly poisoned (saturated), often by reactants (substrates) and reaction products. It was shown that bovine liver catalase is deactivated by the direct action of substrate, the hydrogen peroxide [68]. Indeed, our extracts do contain intrinsically free H_2O_2 responsible for the oxidation mechanisms of phenols (data not shown). This could explain a promotion of the deactivation of catalase in presence of certain extracts when HOCl is added to the system. On the other hand, studies on maritime pine bark extract (PBE, which corresponds to Oligopin®), which is a mixture of bioflavonoids and proanthocyanidins, have demonstrated that PBE dose-dependently inhibited the activity and changed the electrophoretic mobility of catalase under certain conditions. This study also suggested that PBE extract had an effect on catalase activity by binding to the enzyme [48]. Further investigations should be

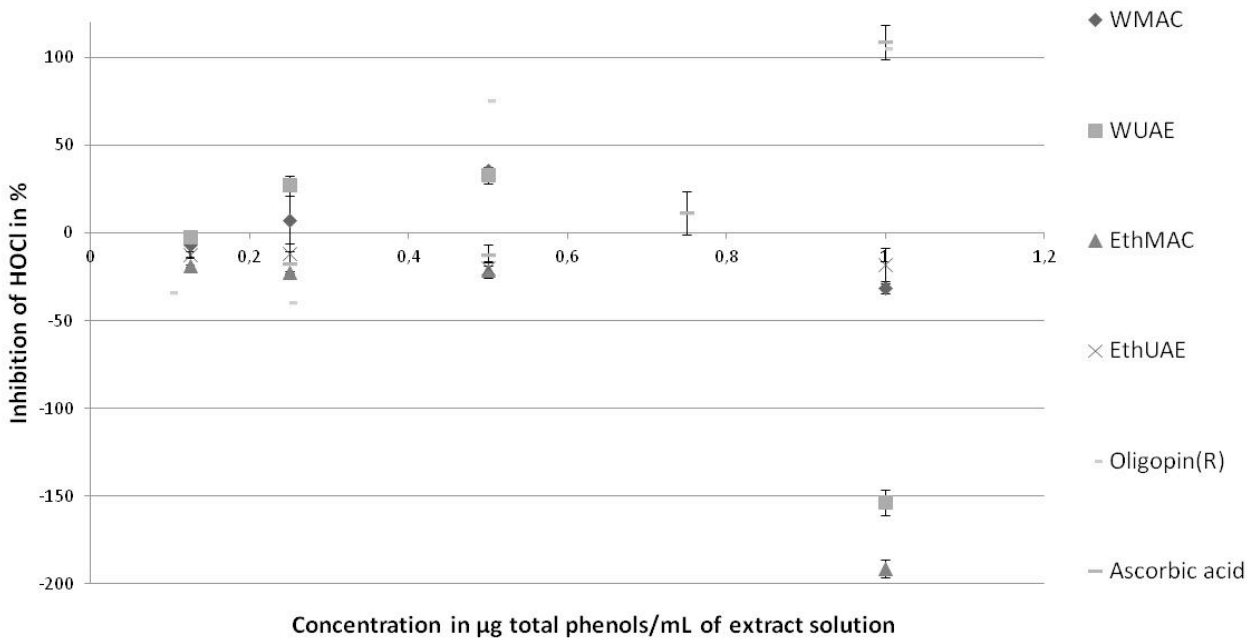

Figure 3: Inhibition percentages of catalase deactivation by HOCl in presence of *C. cornuta* twig extracts.

performed on a proper enzymatic assay of catalase in order to study the mechanism of action of *C.cornuta* twig extracts and determine their selectivity to modulate enzyme activities. Therefore, there could be two phenomena explaining the reduction of catalase activity towards HOCl in the presence of phenolic extracts: contamination of the enzyme by the intrinsically produced H_2O_2 and/or by binding of the proanthocyanidins to the active sites of the enzyme.

C. cornuta twig extracts were also tested against an enzyme that catalyzes the oxidation of its substrate by molecular oxygen and hence generates either intermediate or end product-reactive oxygen species in the enzymatic reaction. Xanthine oxidase (XO) catalyzes the generation of superoxide anion and H_2O_2 by reduction of O_2, while the hypoxanthine and xanthine are oxidized to uric acid. XO is considered an important biological source of superoxide radical involved in the process of oxidative stress [48]. The activities of the various extracts tested at several concentrations of total phenols/ mL of extract solutions were compared with those of the maritime pine bark extract, Oligopin® (DRT, Dax, France), quercetin and curcumin (Sigma Aldrich, St. Louis, MO, USA). The results are presented in Table **4**. All extracts showed an activity against XO higher than Oligopin®. Eth_{MAC} and W_{UAE} extracts were the two most active followed by

Table 4: IC_{50} of *C. cornuta* Twig Extracts Determined to Evaluate their Capacity to Inhibit the Two Oxidation Enzymes:Catalase and Xanthine Oxidase

Extracts and references	Catalase deactivation IC_{50} (µg of total phenols/mL of extract solution)	Xanthine oxidase IC_{50} (µg of total phenols/mL of extract solution)
W_{MAC}	863.3 ± 14.8^{c}	$103.0 \pm 9.3^{b,c}$
W_{UAE}	668.8 ± 16.7^{b}	71.5 ± 29.9^{b}
Eth_{MAC}	$<50^{a}$	13.1 ± 7.2^{a}
Eth_{UAE}	$<50^{a}$	$73.7 \pm 16.4^{b,c}$
Oligopin®	1040.5 ± 30.1^{d}	1040.5 ± 30.1^{d}
Ascorbic acid	$>2000^{e}$	-
Quercetin	-	0.014 ± 0.006^{a}
Curcumin	-	106.2 ± 1.9^{c}

Means with different letters in the same column are significantly different at $p<0.05$ (ANOVA, followed by Duncan test).

EthUAE extract. Between the two aqueous extracts, it can be noticed that the phenolic compounds present in W_{UAE} are significantly more active than those present in W_{MAC} extract. The activity of *C.cornuta* twig extracts increased in a dose-dependence manner. At the highest dose tested (1000 µg total phenols/mL of extract solutions), extracts inhibit 100% of the enzyme while Oligopin® inhibits 43% of the enzyme activity. *C.cornuta* extracts deactivate XO at very low doses in total phenols. It has been demonstrated that maritime pine bark extract (PBE) changes also the electrophoretic mobility of XO and that the inhibition of this enzyme by this proanthocyanidin-rich extract can be explained by a phenomenon of complexation of XO by PBE [48]. Indeed, Eth_{MAC} and W_{UAE} extracts are the richest in proanthocyanidins (Table **1**). Spearman's correlation data presented in Table **6** show a low positive correlation between TP content and the capacity of the extracts to inhibit xanthine oxidase. Considering the various classes of polyphenols, it seems that phenolic acids and proanthocyanidins, more important in ethanolic extracts, are involved in their anti-enzymatic activities as shown by the significant and strong positive correlations determined (*p*-value <0.05). Our results are in agreement with other studies. Indeed, it has already been proved that some hydroxycinnamic acids such as caffeic acid inhibit xanthine-oxidase [69, 70]. Chan *et al*. [69] have pointed out that the molecular structure of the hydroxycinnamic acid has a very important influence on xanthine oxidase inhibition. Proanthocyanidin activity against xanthine oxidase has also been shown in several studies [71]. There is a high variation in the possible mechanisms for enzyme inhibition. Moini *et al*. [48] have demonstrated that proanthocyanidin-rich extract of maritime pine bark (PBE) selectively inhibits xanthine oxidase through binding to the enzyme rather than by the redox activity. On the other hand, we observed to a lesser extent, a negative correlation between flavonoids and xanthine-oxidase inhibition. Lin *et al*. [72] showed the importance of the structure of the flavonoids for the mechanism of xanthine oxidase inhibition, which is related to the binding of these compounds to the active sites of the enzyme. These authors have demonstrated that the 3-substituted hydroxyl benzopyranone ring exhibited weak inhibitory effect, which could be explained by the destabilization of polar hydroxyl stretching into the hydrophobic region of active site of the enzyme and resulted in lowering the binding affinity. It has also been demonstrated that the glycosyl substitution at the C6 position of flavonoid benzopyranone ring also hinders the binding of inhibitors into the active site of xanthine oxidase. It may be possible that some flavonoids contained in *C. cornuta* twig extracts present an inadequate structure for interaction with this enzyme. Further investigation about the structural elucidation of compounds present in this extract are necessary in order to better understand the correlations determined in this research.

3.8. Toxicological Evaluation of *Corylus cornuta* Extracts on Normal Human Kératinocytes

Previous studies have suggested that cultured human keratinocytes may be predictive of irritancy caused by various surfactants in human subjects [73]

Table 5: Toxic Doses of the Extracts Corresponding to 50% (IC_{50}) of Cell Viability at Three Lines of NHK for *C. cornuta* Extracts After 24 and 48 hrs of Exposition of Normal Human Skin Keratinocytes, Determined by MTT and Neutral Red Methods

Extracts	MTT (24h) IC_{50} (µg/mL)	MTT (48h) IC_{50} (µg/mL)	NR (48h) IC_{50} (µg/mL)
W_{MAC}	>1000	734,5	314,4
	>1000	605,5	124
	>1000	>1000	236,1
W_{UAE}	895,3	>1000	399,4
	>1000	897,5	213,4
	>1000	>1000	248,9
Eth_{MAC}	905,4	561,8	> 62,5 a
	941,6	715,5	> 62,5
	952,3	571,1	n.d.
Eth_{UAE}	840,4	518,6	> 62,5
	730,2	546,1	> 62,5
	712,6	507,9	n.d.

and the data provided by this model have shown a good correlation with *in vivo* skin irritation [74]. The toxicological properties of the most efficient antioxidant extracts (W_{MAC} and W_{UAE}) were evaluated on normal human keratinocytes during 24 and 48 hours by MTT and after 48h for Neutral Red method through the determination of the mean toxicity value (IC_{50}). Toxicological properties of the vehicle used (cell culture medium containing 1 % ethanol in the case of ethanolic extracts) was not found to have any significant effect on toxicity of keratinocytes by MTT method (data not shown). Problems of solubility of ethanolic extracts in the medium do not permit the right calculation of their respective IC_{50} by Neutral Red Method after 48h exposition. The results (Table **5**) show that in general, aqueous extracts are the least toxic on NHEK (p< 0.05) after 48h while ethanolic extracts are the most toxic. It is interesting to observe the effect of ultrasounds on the preferential extraction of molecules, since the toxicity of the extracts has been shown to depend on the solvent chosen for extraction. Therefore, it seems that after 48 h, the major contribution to the toxicity could be attributed to the presence of polyphenolic metabolites as result of interaction with keratinocytes. Indeed, we observed a decrease of the IC_{50} after 48h exposition compared to 24h exposition, meaning that *C. cornuta* extract phenolic-oxidation products caused by peroxidase activity present in kératinocytes contribute to mitochondrial toxicity [39]. Spearman's correlation data presented in Table **6** have been calculated only with MTT assay results on the basis of IC_{50} obtained after 48h exposition to NHEK. The results show that there is a significant negative correlation between MTT IC_{50} and TP content (p-value < 0.05), meaning that the more TP content, the higher is the cytotoxicity of the extract. No correlations were found with phenolic acids with PAs neither. On the other hand, a significant positive correlation was found between flavonoid content and the extract cytotoxicity. Flavonoids present in *C. cornuta* twig aqueous extracts are responsible of their high antioxidant/antiradical properties and their low cytotoxicity.

3.9. Active Dose vs Toxic Dose

In order to know how safe the extracts were for keratinocytes at their antioxidant/antiradical active concentrations, we calculated the relationship between

Table 6: Spearman's Correlation Coefficients Obtained Between Antioxidant/Antiradical and Anti-Enzymatic Activities as well as Cytotoxicity on NHK and the Polyphenolic Composition of *C. cornuta* Twig Extracts

Extracts activity	TP	THCA	TFlav	PAs
Superoxide	R= -0.75 p-value = 0.007353	R = 0.27 p-value = 0.2330	R= 0.88 p-value = 0.0001922	R = -0.74 p-value = 0.00817
Hydroxyle	R^2= -0.59 p-value = 0.04884	R = 0.13 p-value = 0.7435	R = 0.77 p-value = 0.02139	R = 0.12 p-value = 0.7756
Peroxyde Hydrogène	-	-	-	-
Peroxyl	R = -0.73 p-value = 0.009052	R = -0.04 p-value = 0.9037	R = 0.67 p-value = 0.02044	R = 0.02 p-value = 0.9562
Nitric oxide	R= -0.67 p-value = 0.02044	R = 0.14 p-value = 0.6672	R = 0.52 p-value = 0.08388	R = 0.46 p-value = 0.03803
Hypochlorous acid	R= -0.89 p-value = 0.03333	R = -0.28 p-value = 0.3331	R = -0.15 p-value = 0.6158	R = 0.15 p-value = 0.6511
Lipidic peroxidation	R = -0.94 p-value < 2.2e-16	R = -0.35 p-value = 0.256	R = 0.61 p-value = 0.003702	R = -0.35 p-value = 0.266
TEAC	R^2= -0.29 p-value = 0.3543	R = -0.21 p-value = 0.5135	R = 0.53 p-value = 0.0793	R = -0.20 p-value = 0.5281
DPPH Scavenging	R = -0.79 p-value = 0.003617	R = -0.76 p-value = 0.005897	R = 0.86 p-value = 0.0004433	R = -0.77 p-value = 0.005253
Catalase	-	-	-	-
Xanthine oxidase	R= 0.57 p-value = 0.05903	R = 0.76 p-value = 0.005897	R = -0.64 p-value = 0.02795	R = 0.86 p-value = 0.0005971
IC_{50} MTT assay (48h)	R= -0.77 p-value = 0.003090	R= 0.02 p-value = 0.948	R= 0.61 p-value = 0.03688	R= -0.01 p-value = 0.9653

MTT IC_{50} values and the antioxidant capacity (IC_{50}). We found that antiradical concentrations of *C.cornuta* aqueous extracts for scavenging H_2O_2 and ROO• were approximately 2.4-6.2 times lower than cytotoxic concentrations whereas their antioxidant/antiradical concentrations for scavenging O_2^-• and NO• were approximately 12.6-125.8 times lower than cytotoxic concentrations. This last estimation pointed out the strong effect of *C.cornuta* twig extracts on reactive species involved in inflammatory processes. On the contrary, the effective activity of the extracts to scavenge OH•was obtained at toxic concentration for human keratinocytes. On the other hand, the concentrations of aqueous extracts effective for inhibiting lipidic peroxidation were approximately 8.7-27.9 times lower than their cytotoxic concentrations. Considering ethanolic extracts, the concentrations required for inhibiting xanthine oxidase were approximately 7.6-43.8 times lower than their cytotoxic concentrations. These extracts could be therefore good candidates for the development of therapeutic drugs to treat gout. These estimations should not be, however, considered as completely predictive of the effectiveness of extracts in cell systems at concentrations lower than IC_{50}. Chemical reactivity is only a part of bioactivity and in cellular context other factors such as the activation/deactivation of polyphenolic compounds as a consequence of metabolism and the interaction with endogenous antioxidants should be considered.

4. CONCLUSION

Although there are several reports on the traditional uses of *C.cornuta* twigs in external applications or about how the twig extracts (infusions) were applied remedies to soothe and relieve various human diseases (arthritis, teeth pain, gastric ulcers etc.) and treat skin problems, there were no scientific evidences about their bioactivity and their toxicological properties. The present study, for the first time, demonstrates the antioxidant/antiradical and anti-enzymatic properties as well as the toxicological properties of *C.cornuta* twig extracts rich in polyphenols, on Normal Human Keratinocytes. *C.cornuta* twigs are rich in extractives and a large proportion of them are polyphenolic compounds. The modern method of extraction assisted by ultrasound has proven to be more effective with ethanol as solvent than with water, and high extraction yields and total phenol contents were achieved with this extraction. Even though lower yields were obtained by water extraction assisted by ultrasounds it seems that this method permitted to target certain classes of polyphenols such as flavonoids or proanthocyanidins. Aqueous extracts were determined to have high content of total phenols and flavonoids and low toxicity on NHK. Our results indicate clearly an important role of flavonoids in radical scavenging, in particular against superoxide and hydroxyl radicals, as well as in the inhibition of lipid peroxidation. The water extracts of *C. cornuta* twigs were particularly determined to be more effective than ethanolic extracts against reactive species involved in inflammatory processes. This last property should be further investigated in order to establish the relevance of these results for the treatment of symptoms claimed by the traditional remedies based on water extracts of *C. cornuta* twigs. In further investigations, the isolation and identification of compounds present in the aqueous extract will be performed, along with various studies on the anti-inflammatory potential of *C. cornuta* aqueous extract.

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