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Phenolic Composition and Antioxidant Properties of Poplar Bud (*Populus nigra*) Extract: Individual Antioxidant Contribution of Phenolics and Transcriptional Effect on Skin Aging

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ABSTRACT: The *Populus* species possess great potential for therapeutical applications, especially for their known anti-inflammatory properties. The antioxidant properties of propolis, a hive product collected by honey bees mainly from poplar bud exudates, suggest that poplar buds also possess antioxidant properties. Here is reported the characterization of the antioxidant properties of an aqueous poplar bud (*Populus nigra*) extract. It presented a high total phenolic content, and moderate antioxidant properties as determined by ORAC assay. The main phenolic compounds identified were phenolic acids and flavonoid aglycons. These phenolic compounds were analyzed by ORAC assay for their individual antioxidant activity, in order to determine the major contributors to the total antioxidant activity of the extract. Thanks to their high antioxidant activity, caffeic and *p*-coumaric acids were identified as the major antioxidant components. Representing only 3.5% of its dry weight, these compounds represented together about 50% of the total antioxidant activity of the extract. The antioxidant properties of poplar bud extract and the phenolic compounds identified were also analyzed by cellular antioxidant activity assay (CAA), which was weakly correlated with ORAC assay. The transcriptional effect of poplar bud extract on skin aging was evaluated in vitro on a replicative senescence model of normal human dermal fibroblasts, using a customized DNA macroarray specifically designed to investigate skin aging markers. Among the detected genes, poplar bud extract significantly regulated genes involved in antioxidant defenses, inflammatory response and cell renewal. The collective antioxidant properties and transcriptional effect of this extract suggest potential antiaging properties which could be utilized in cosmetic and nutraceutical formulations.

KEYWORDS: *Populus nigra*, poplar bud, antioxidant, ORAC, CAA, cellular antioxidant activity, DCFH-DA, skin aging

INTRODUCTION

The genus *Populus* belongs to the Salicaceae family and comprises more than one hundred species which are distributed in temperate and subtropical regions.[1] Several species of *Populus* have been used traditionally in medicine,[2,3] especially for their anti-inflammatory properties.[4,5] Poplar buds are coated with a viscous substance, an exudate, which was reported to contain different varieties of phenolic compounds, depending on the species studied: terpenoids, flavonoid aglycons and their chalcones and phenolic acids and their esters.[6−8] Among the *Populus* species, the black poplar (*Populus nigra*) is widely distributed in Europe. The chemical characterization of its bud exudate has allowed the identification among the flavonoid aglycons of some flavanones such as pinocembrin and pinostrobin, some flavonols such as galangin, quercetin and kaempferol, some flavones such as chrysin and apigenin,[9,10] and some esters of phenolic acids.[11] Such compounds have also been reported in propolis,[12] a hive product collected by honeybees from tree buds, especially poplar buds.[13] Propolis has long been used in popular medicine to cure many diseases,[14] owing to its antimicrobial, anti-inflammatory and antioxidant properties.[15]

In recent years, functional foods (nutraceuticals) have attracted growing attention because of consumers' increasing concerns about their health, which has spurred greater research effort into such foods. Antioxidant nutraceuticals have received particular attention, as oxidative stress, defined as the imbalance between oxidants and antioxidants in favor of the oxidants,[16] is known to be involved in various diseases, as well as in aging. Indeed, the free radical theory of aging envisions that aging derives from the accumulation, over a lifetime, of oxidative damage to cells resulting from excess reactive oxygen species, which are produced as a consequence of aerobic metabolism.[17] Skin, as the outermost barrier of the body, directly interacts with a harmful oxidative environment and thus is a major target of aging.[18] Skin possesses extremely efficient defense mechanisms such as antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) and nonenzymatic antioxidant molecules (vitamin E, vitamin C, glutathione, ubiquinone), but it has been demonstrated that these defenses decline during aging[19] and need to be supported by an intake of antioxidant compounds, which possess beneficial effects thanks to their ability to neutralize reactive oxygen species and to limit the inflammation they cause.[20]

Although the antioxidant properties of propolis have been intensively investigated and have been suggested to be due, at

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least in part, to poplar bud phenolic compounds, the antioxidant properties of poplar buds have not been directly studied. Thus, an aqueous extract of poplar buds was investigated for its phenolic composition and its antioxidant properties using ORAC and cellular antioxidant activity assay (CAA). The main phenolic compounds were identified by MS and NMR and quantified, and their individual antioxidant activity was determined using ORAC assay in order to identify the major contributors to total antioxidant activity of the extract. The cellular antioxidant activity of the phenolic compounds of poplar bud extract was analyzed using CAA assay. In addition, to highlight a potential application of this antioxidant extract in nutraceutical formulations, the transcriptional effect of poplar bud extract on skin aging was investigated on a replicative senescence model of normal human dermal fibroblasts (NHDF), using a customized DNA macroarray containing 149 skin-related genes covering dermal structure, cell renewal, inflammatory response and oxidative stress mechanisms.

MATERIALS AND METHODS

Biological Materials and Chemicals. Poplar buds (*Populus nigra,* Salicaceae, Romania) were obtained from the Biolandes company's plant collection, and were extracted industrially. 100 kg of plant raw material was extracted by water at 50 °C under agitation. After filtration, the solution was concentrated at 50 °C under vacuum and then dried by spray drying.

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), phosphate buffer, caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, di-*O*-methylcaffeic acid, cinnamic acid, salicin, sodium carbonate, Folin−Ciocalteu reagent, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and phosphate buffer saline were purchased from Sigma-Aldrich (France). Pinocembrin was obtained from Extrasynthese (France), and pinobanksin and pinobanksin 5-methyl ether were purified by preparative high performance liquid chromatography (HPLC). For cellular antioxidant assay, normal human dermal fibroblasts were obtained from face skin surgery (Promocell, France) and RPMI medium, fetal bovine serum, L-glutamine, penicillin and streptomycin were purchased from Sigma-Aldrich (France). For macroarray experiments, normal human dermal fibroblasts were isolated from healthy skin obtained from plastic surgery and DMEM medium, fetal bovine serum, L-glutamine, penicillin and streptomycin were purchased from Fisher Scientific (Loughborough, Leicestershire, U.K.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrasolium bromide (MTT) was also obtained from Fisher Scientific (Loughborough, Leicestershire, U.K.).

Total Phenolic Content Determination. The total phenolic content of poplar bud extract was determined according to the Folin−Ciocalteu method, using caffeic acid as standard. Twenty microliters of aqueous solution of plant extract and 80 μL of sodium carbonate solution (7.5% in deionized water) were added to 100 μL of Folin−Ciocalteu reagent (diluted 10-fold in deionized water) in a 96-well plate. A blank sample and six calibration solutions of caffeic acid (0.625 to 20 μg/mL, final concentration) were analyzed under the same conditions. After incubation for 1 h at room temperature, the absorbance was measured at 760 nm using a Fluostar Optima plate reader (BMG Labtech, Germany). All determinations were carried out in triplicate, and results were expressed as mg caffeic acid equivalent/g of extract.

Free Radical Scavenging Capacity Determination by ORAC Assay. The ORAC assay was applied as described previously.[21] The reaction was carried out in 75 mM phosphate buffer (pH 7.4) in a 96-well plate. Thirty microliters of poplar bud extract or pure phenolic compound solutions and 180 μL of fluorescein solution (70 nM final concentration) were mixed and preincubated for 5 min at 37 °C. 90 microliters of APPH solution (12 mM final concentration) was then added, and the fluorescence was recorded for 60 min at excitation and emission wavelengths of 485 and 520 nm respectively. The 10 identified phenolic compounds were mixed in proportions determined by their quantification and analyzed under the same conditions. A blank sample and six calibration solutions of Trolox (0.1 to 4 μM, final concentration) were also tested in each assay. All samples were analyzed in triplicate. Area under curve (AUC) was calculated for each sample by integrating the fluorescence curve. Net AUC was calculated by subtracting the AUC of the blank. The regression equation between net AUC and Trolox concentration was determined, and ORAC values were expressed as equivalent concentration of Trolox.

Free Radical Scavenging Capacity Determination by CAA Assay. The cellular antioxidant assay was applied according to the method of Wolfe,[22] slightly modified. Normal human dermal fibroblasts (NHDF) were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. The effect of poplar bud extract (25 to 200 μg/mL in culture medium) on NHDF viability was determined using the MTT assay, as described previously.[23] The cells were used between passages 7 and 10 and were seeded in 96-well plates at a rate of 10^4 cells per well for 24 h. NHDF were treated with 100 μL of poplar bud extract or pure phenolic compounds solutions (25 to 200 μg/mL and 25 to 200 μM final concentrations respectively) and 100 μL of DCFH-DA solution (25 μM final concentration) prepared in culture medium. After 1 h of incubation at 37 °C, the treatment medium was removed, the cells were washed twice with 200 μL of PBS, and then 100 μL of AAPH solution was added (250 μM final concentration). The fluorescence was recorded every 30 min for 180 min at wavelengths of excitation and emission of 485 and 520 nm respectively. A blank without sample and without oxidant was analyzed in order to evaluate the fluorescence due to endogenous production of free radicals. In addition, a control without antioxidant was analyzed under the same conditions. All determinations were carried out in triplicate. Area under curve (AUC) was calculated for each sample by integrating the fluorescence curve. Net AUC was calculated by subtracting the AUC of the blank. Radical scavenging activity was calculated using the following formula:

$$\%\ \text{inhibition} = 100 - [(\text{net AUC}_{\text{sample}} \times 100)/(\text{net AUC}_{\text{control}})]$$

Characterization of Poplar Bud Extract Phenolic Compounds. The phenolic profile of poplar bud extract was analyzed by reverse-phase analytical HPLC using a Varian Prostar HPLC. The separation of phenolic compounds was performed using a Prontosil C18 reverse phase column (250 mm × 4 mm, 5 μm particle size), protected with an Ultrasep C18 guard column (Bischoff chromatography, Germany). Poplar bud extract was injected (100 μg), and the elution (1 mL/min) was performed using a solvent system comprising solvents A (0.1% trifluoroacetic acid (TFA) in water) and B (0.1% TFA in methanol) mixed using a gradient starting with 0% B and linearly increasing to 72% B in 50 min. Chromatographic data were acquired at 200, 280, and 325 nm. The isolation of phenolic compounds was performed by preparative HPLC using a Prontosil C18 reverse phase column (250 mm × 20 mm, 5 μm particle size), protected with an Ultrasep C18 guard column (Bischoff chromatography, Germany). Poplar bud extract was injected (50 mg), and the elution (15 mL/min) was performed using the solvent system and the conditions described above. The eluted compounds were manually collected according to the visualization of the UV profile. The isolated compounds were analyzed for their antioxidant activity in order to lead the characterization to the active compounds. Purified phenolic compounds were identified by

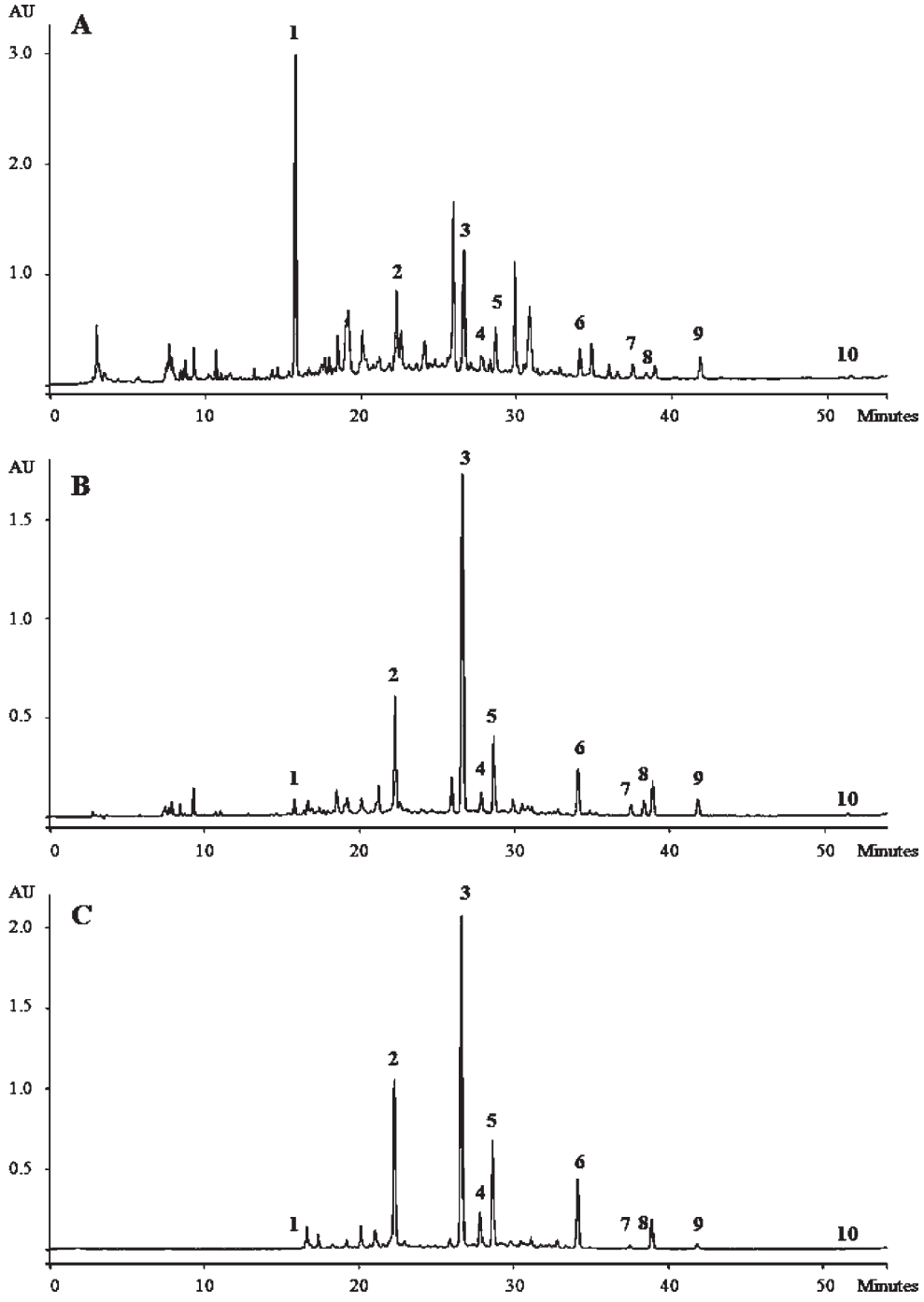
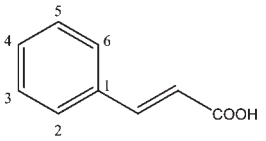
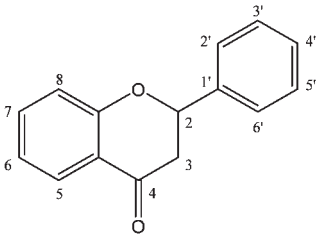
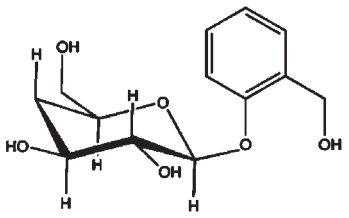


Figure 1. HPLC−DAD chromatograms of poplar bud extract at 200 nm (A), 280 nm (B) and 325 nm (C). For compound assignment see Table 2.

mass spectrometry analysis and confirmed by ^{1}H NMR. MS analysis was performed using an Esquire 3000 plus MS (Bruker Daltonics, Germany). The ion trap mass spectrometer was equipped with an electrospray ionization (ESI) source operated in negative ion mode. The electrospray capillary voltage was set to 3000 V with a nebulizing gas flow rate of 10 L/min and a drying gas temperature of 350 °C. MS data were acquired in the scan mode (mass range m/z 150−1200). NMR analysis was performed using a Bruker 600 MHz at a temperature of 300 K. The samples were dissolved in deuterated methanol (CD_3OD) and the structure of compounds was obtained by ^{1}H NMR. The spectra of purified phenolic compounds were compared with those of commercial standards when available. Otherwise, the chemical shifts of purified phenolic compounds were compared with those reported in the literature. The phenolic compounds identified were quantified by analytical HPLC by the external standard method using calibration solutions of standards (0.05 to 5 μg) at 200 nm for salicin, 280 nm for flavonoid aglycons and cinnamic acid, and 325 nm for other phenolic acids.

Gene Expression of Normal Human Dermal Fibroblasts (NHDF). NHDF were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin and maintained at 37 °C with 5% CO_2 in a humidified atmosphere. For macroarray experiments, cells were used in either passage 7 or 17, referred to as "normal" and "aged" cells,

Table 1. Structure of Poplar Bud Extract Phenolic Compounds

Compound	Substitution
Phenolic acids	
Caffeic acid	3,4-OH
Para-coumaric acid	4-OH
Ferulic acid	3-OCH_3, 4-OH
Isoferulic acid	3-OH, 4-OCH_3
Di-O-methylcaffeic acid	3,4-OCH_3
Cinnamic acid	-
Flavonoids	
Pinobanksin	3,5,7-OH
Pinobanksin 5-methyl ether	3,7-OH, 5-OCH_3
Pinocembrin	5,7-OH
Salicin	

respectively. Normal cells were untreated. Aged cells were either treated with poplar bud extract (2 μg/mL) or untreated. Cells were cultivated for 72 h, and each experimental condition was performed in triplicate. The total RNA of each cell culture was extracted using a commercially available RNA extraction mixture, TriReagent, in accordance with the manufacturer's instructions. The concentration of RNA, its purity and its quality were estimated using an Agilent 2100 BioAnalyzer. Macroarrays were developed by BIOalternatives SA (Gençay, France) and were prepared by spotting cDNAs specific markers of interest on nylon membranes using an array spotter (Piezorray, PerkinElmer, France). The multiple cDNA ^{33}P-labeled targets were prepared by direct reverse transcription of NHDF-extracted RNA, using [^{33}P]-dATP and oligodT. These labeled targets were hybridized to the specific cDNA probes covalently fixed to the macroarrays containing 149 genes (2 spots per gene). After extensive washing, the membranes were analyzed by direct quantification of spot radioactivity using a "Cyclone" PhosphorImager (Packard Instruments, France). The results were expressed in relative expression units (RE), radioactivity average of the double spot for each gene, after correction of the background noise and the differences in the labeling of the probes. The correction was based on the intensity of the housekeeping genes from the different membranes. In this experiment, a gene was considered to be expressed significantly when its RE was at least 2. Gene expression levels of normal and treated aged cells were expressed as percentage of untreated aged cell gene expression level, defined as 100%. The significance limit was set at <65% for repression and >140% for upregulation.

Statistical Analysis. Results were expressed as means ± standard deviation (SD) of three measurements. Statistical analysis was performed using Student's t test, and $P < 0.1$ was considered to be significant. Correlations among data obtained were calculated using the MS Excel software correlation coefficient statistical option.

Table 2. Phenolic Composition of Poplar Bud Extract

peak	compound	RT (min)	λ_{max} (nm)	content (% of dry wt)
1	salicin	16.6	200	1.79 ± 0.07
2	caffeic acid	22.8	325	1.64 ± 0.07
3	*p*-coumaric acid	26.8	325	1.88 ± 0.08
4	ferulic acid	28.1	325	0.24 ± 0.01
5	isoferulic acid	28.9	325	0.71 ± 0.03
6	di-*O*-methylcaffeic acid	33.9	325	0.67 ± 0.03
7	pinobanksin 5-methyl ether	37.3	280	0.37 ± 0.06
8	cinnamic acid	38.2	280	0.11 ± 0.05
9	pinobanksin	40.7	280	0.98 ± 0.10
10	pinocembrin	49.7	280	0.10 ± 0.01
	total			8.49

RESULTS AND DISCUSSION

Phenolic Composition and Antioxidant Properties of Poplar Bud Extract. Aqueous poplar bud extract presented a high total phenolic content of about 180 mg caffeic acid equivalent/g of sample and an antioxidant activity of about 2700 μmol of Trolox/g as determined by the ORAC assay, which is moderate in comparison with previously analyzed aqueous plant extracts.[24] HPLC separation of the phenolic compounds of poplar bud extract is presented in Figure 1, and their identification is shown in Table 1. The phenolic composition of poplar bud extract is presented in Table 2. About 8.5% of the dry weight of the extract was characterized, corresponding to the identification of 10 phenolic compounds. Poplar bud extract was mainly composed of phenolic acids (caffeic, *p*-coumaric, ferulic, isoferulic, di-*O*-methylcaffeic and cinnamic acids) (5.2%), followed by salicylates (salicin) (1.8%) and flavonoid aglycons (pinobanksin 5-methyl ether, pinobanksin and pinocembrin) (1.5%). These compounds were identified by MS and NMR analyses, using commercial standards for comparison when available. Their MS characteristics and NMR chemical shifts were compared with those reported in the literature.[25−28] Such phenolic compounds were earlier identified in poplar-type propolis[12] and/or in the buds exudates of others *Populus* species,[6,7] but almost all are reported for the first time specifically in *Populus nigra* bud exudates. Salicin is a salicylate that is a characteristic compound of the Salicaceae family.[29] The major phenolic compounds, identified as *p*-coumaric acid, salicin and caffeic acid, represent together more than 5% of the dry weight of the extract.

Antioxidant Activity of Poplar Bud Phenolic Compounds and Relationship with Their Structure. The ORAC values of the phenolic compounds identified are presented in Table 3.

Table 3. Antioxidant Activities of Poplar Bud Extract Phenolic Compounds and Individual Antioxidant Contribution Determined by ORAC Assay

		antioxidant activity		
peak	compound	(μmol of Trolox/g)	(μmol of Trolox/μmol)	antioxidant contribution (%)
1	salicin	43 ± 2	0.01 ± 0.00	0.03 ± 0.00
2	caffeic acid	41639 ± 233	7.50 ± 0.04	24.94 ± 0.14
3	*p*-coumaric acid	38429 ± 69	6.31 ± 0.01	26.39 ± 0.05
4	ferulic acid	23836 ± 327	4.63 ± 0.06	2.09 ± 0.03
5	isoferulic acid	30821 ± 132	5.98 ± 0.03	7.99 ± 0.03
6	di-*O*-methylcaffeic acid	0	0.00	0.00
7	pinobanksin 5-methyl ether	6362 ± 205	1.82 ± 0.06	0.87 ± 0.03
8	cinnamic acid	0	0.00	0.00
9	pinobanksin	7330 ± 138	2.00 ± 0.04	2.63 ± 0.05
10	pinocembrin	3957 ± 346	1.01 ± 0.09	0.14 ± 0.01
	total			65.08
	mix of compounds	20083 ± 667		62.35

Overall, the phenolic acids presented a higher antioxidant activity than the flavonoid aglycons (about 4−7 and 1−2 μmol of Trolox/μmol respectively), except for nonhydroxylated phenolic acids, which showed no antioxidant properties. Indeed, the antioxidant capacity of phenolic compounds is directly attributed to their structure. Several studies of structure−antioxidant activity relationship have shown that the number and the position of hydroxyl groups on the phenolic rings are determining parameters of the antioxidant potential.[30,31] For phenolic acids, it was demonstrated that compounds presenting a pyrogallol unit (three hydroxyl groups on the phenol ring) exhibited higher antioxidant properties than those presenting a catechol unit (two hydroxyl groups on the phenolic ring).[32] As expected, caffeic acid (3,4-dihydroxycinnamic acid) presented a higher antioxidant capacity than *p*-coumaric acid (4-hydroxycinnamic acid) (7.50 and 6.31 μmol of Trolox/μmol respectively), and cinnamic acid and di-*O*-methylcaffeic acid (3,4-dimethoxycinnamic acid) exhibited no antioxidant activity, owing to their lack of phenolic hydroxyl groups. Substitution of the 3- or 4-hydroxyl group of caffeic acid by a methoxy group as in ferulic acid (3-methoxy-4-hydroxycinnamic acid) and isoferulic acid (3-hydroxy-4-methoxycinnamic acid) seemed to decrease the antioxidant activity of the compound (4.63 and 5.98 μmol of Trolox/μmol respectively), which is consistent with the nonactivity of di-*O*-methylcaffeic acid, which only presents methoxy groups. These results are not consistent with findings obtained in previous studies using DPPH or ABTS assays, where caffeic acid was reported to have a lower activity than *p*-coumaric, ferulic and isoferulic acids,[30,31] but are consistent with previous studies using ORAC assay.[33,34] This discrepancy in findings obtained with different methods could be due to the fact that, unlike DPPH and ABTS assays, the ORAC assay takes into account the kinetic action of antioxidants. With regard to flavonoids, it has been reported that one of the essential features of the antioxidant potential of flavonoids is the presence of 3- and 5-hydroxy groups adjacent to the 4-oxo structure.[35] As expected, pinobanksin (3,5,7-trihydroxyflavanone) presented the highest antioxidant capacity, followed by pinobanksin 5-methyl ether (3,7-dihydroxy-5-methoxyflavanone), in which the 5-hydroxyl group is substituted by a methoxy group, and pinocembrin (5,7-dihydroxyflavanone), owing to the lack of the 3-hydroxy group (2.00, 1.82, and 1.01 μmol of Trolox/μmol respectively). As for phenolic acids, the presence of hydroxyl groups on the phenolic rings is a determining parameter of the antioxidant potential of phenolic glucosides.[36] Thus, salicin, which presented no phenolic hydroxyl groups, presented almost no antioxidant activity (0.01 μmol of Trolox/μmol).

Individual Antioxidant Contribution of Poplar Bud Extract Phenolic Compounds. Several studies have been conducted to correlate the phenolic composition of plant extracts with their antioxidant properties, but very few have identified the main antioxidant contributors of these extracts. To identify the compounds responsible for the antioxidant activity of a plant extract, one should consider not only their own antioxidant activity but also their abundance in the extract. Identified phenolic compounds were analyzed for their antioxidant activity, not only individually but also mixed together in the proportions found in poplar bud extract, in order to determine the possible synergistic or antagonistic activities between them. The antioxidant contributions of the phenolic compounds identified are presented in Table 3. The sum of the individual antioxidant contributions of the phenolic compounds identified was 65%. The mix had an antioxidant potential of about 20000 μmol of Trolox/g, representing an antioxidant contribution of 62%, which means that the 10 compounds presented the same antioxidant activity alone or mixed with others in solution. This result confirms that the antioxidant activities of the phenolic compounds identified can be added. Thanks to the bioguided approach, more than 60% of the total antioxidant activity of poplar bud extract was characterized, with the identification of only 8.5% of the dry weight of the extract. Since phenolic acids are the most active and the most abundant compounds in poplar bud extract, they represent the major part of the total antioxidant capacity of the extract (61%) unlike flavonoids, which represent only 4% of the extract's activity. Among all compounds identified, caffeic and *p*-coumaric acids had the greatest antioxidant potential. Indeed, although these compounds are each present at about 2% in the extract, they represent together more than 50% of the total antioxidant activity of poplar bud extract. On the other hand, salicin, which also proved to be a major phenolic compound of poplar bud extract, presented almost no antioxidant activity so it contributed very little to the total antioxidant activity of the extract (0.03%).

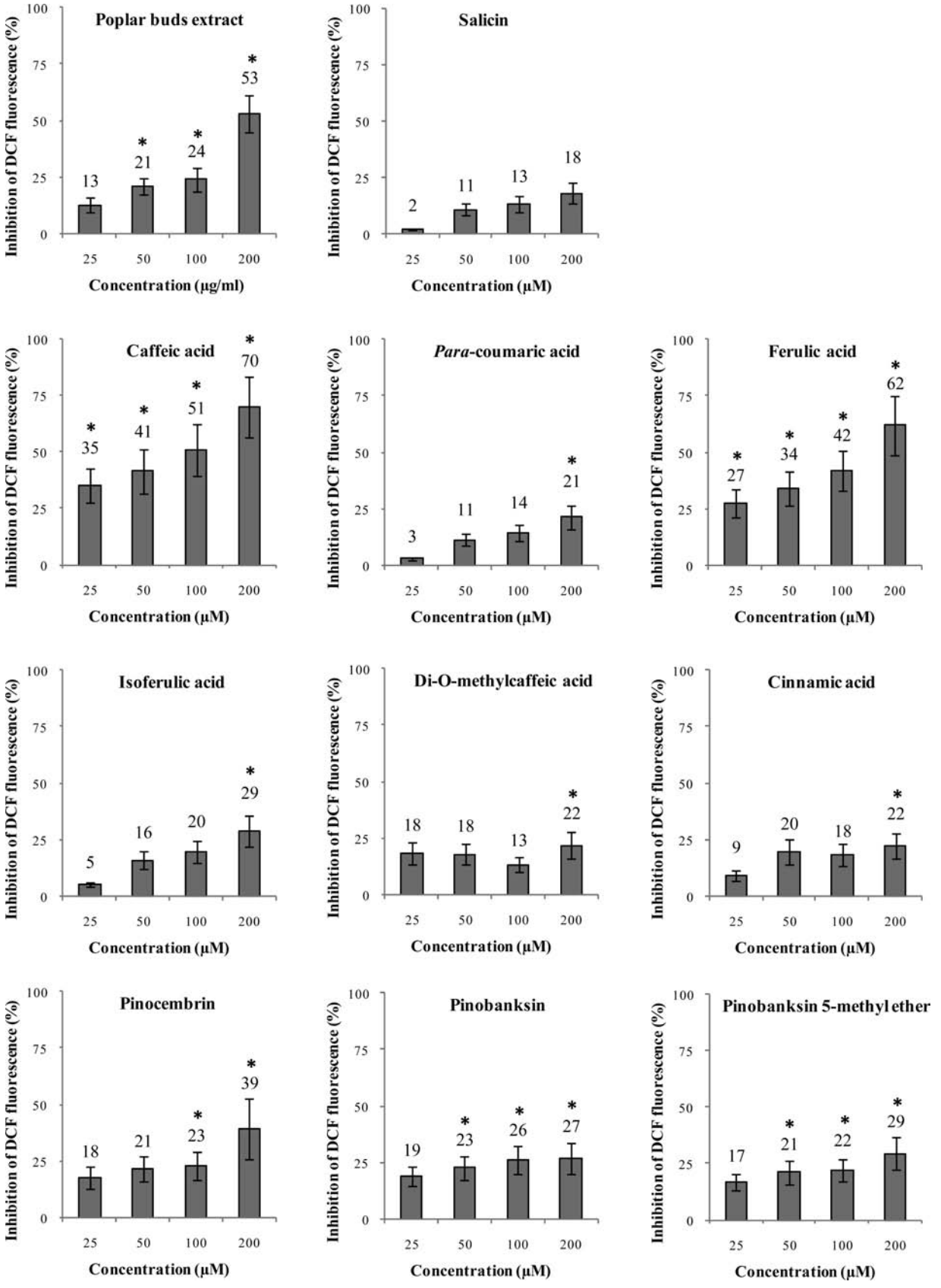


Figure 2. Cellular antioxidant activities of poplar bud extract and its phenolic compounds as determined by CAA assay. * indicates values statistically different from control experiments ($P < 0.1$).

Table 4. Significant Modulation of NHDF Gene Expression by Poplar Bud Extract

gene	role	untreated normal fibroblasts (%)	treated aged fibroblasts (%)
catalase (cat)	protective antioxidant	↑+69	↑+130
chemokine (C−C motif) ligand 5 (CCL5)	inflammatory response	↓−65	↓−39
Krupper-like factor 10 (KLF10)	cell renewal	↑+57	↑+86
E2F-4 transcription factor (E2F4)	cell renewal	↑+110	↑+151
EGF response factor 1 (ZFP36L1)	cell renewal	↑+75	↑+103

Cellular Antioxidant Activities of Poplar Bud Phenolic Compounds and Correlation with ORAC Values. In addition to the antioxidant properties determined by the chemical ORAC assay, poplar bud extract and phenolic compounds identified were analyzed using the CAA assay (cellular antioxidant activity assay), using DCFH-DA as the probe. DCFH-DA is a commonly used indicator of endogenous oxidative stress.[22] It diffuses through the cell membrane and, once within the cell, is deacetylated by cellular esterases to form DCFH, which is trapped within the cell owing to its more polar nature. Cells are then treated with AAPH, which is able to diffuse into cells and to decompose spontaneously to form peroxyl radicals. These peroxyl radicals allow the production of more radicals and oxidize the intracellular DCFH to the fluorescent DCF. Pretreatment of cells with antioxidants prevents the oxidation of DCFH and reduces the formation of DCF. As the antioxidant treatment is removed prior to the oxidant treatment, this assay only takes into account the antioxidant compounds which are incorporated in cells.

The safety of poplar bud extract on NHDF was checked at the different concentrations tested (data not shown). The cellular antioxidant activities of poplar bud extract and the phenolic compounds identified are presented in Figure 2. Poplar bud extract and all tested compounds were shown to reduce radical formation at various doses, but their behavior was very different. Poplar bud extract presented strong cellular antioxidant properties as it inhibits up to 50% of radical production at the concentration of 200 μg/mL. Among the phenolic compounds, caffeic and ferulic acids presented the highest antioxidant activities with the inhibition of up to more than 60% of radical production at 200 μM, followed by pinocembrin, which showed radical scavenging of up to 40%. Salicin, *p*-coumaric, isoferulic, di-*O*-methylcaffeic and cinnamic acids, as well as pinobanksin and pinobanksin 5-methyl ether, presented lower antioxidant activities with inhibition of up to 30% of radical production at 200 μM. Poplar bud extract and almost all pure phenolic compounds showed a dose-dependent antioxidant activity (correlation coefficients R from 0.936 to 0.999, data not shown). Caffeic and ferulic acids, which were the most active compounds with this assay, presented a real dose−antioxidant effect (correlation coefficients R of 0.999 and 0.998 respectively), which suggested a good incorporation of these compounds within the cells. Conversely, di-*O*-methylcaffeic acid showed the lowest dose−effect (correlation coefficient R of 0.434). It presented almost the same radical scavenging activity of about 20% whatever the concentration tested, which suggests that the maximum incorporation of this compound is achieved at a concentration of 25 μM.

The radical scavenging activities of pure phenolic compounds determined by CAA assay differed from those obtained with ORAC assay, which is consistent with previous studies.[37,38] The correlation coefficients R between ORAC values (μmol of Trolox/μmol) and CAA values (inhibition % at concentrations of 25, 50, 100, and 200 μM) were 0.271, 0.429, 0.578, and 0.578 respectively (data not shown). These values are lower than previously reported correlation coefficients between ORAC and other antioxidant assays.[24] This discrepancy could be due to the biological aspect of the CAA assay. It considers the uptake and metabolism of the antioxidant compounds, and their efficiency of protection against peroxyl radicals under physiological conditions. Thus, some phenolic compounds which were totally inefficient in the ORAC assay, such as di-*O*-methylcaffeic acid and cinnamic acid, exhibited a low but existent antioxidant activity with CAA assay. This suggests that these compounds would not be effective against peroxyl radicals but would be against other types of radicals formed as a consequence of AAPH-induced oxidation, or eventually that these compounds exert their antioxidant activity through a mechanism other than free radical scavenging. Although ORAC and CAA assays were not well correlated, caffeic acid, which was the most active compound identified in poplar bud extract with the ORAC assay, was also the most active compound with the CAA assay. Similarly, salicin was the least active compound in both tests.

NHDF Gene Expression Modulation by Poplar Bud Extract. The transcriptional effect of poplar bud extract on NHDF was evaluated using a customized DNA macroarray specifically designed to investigate skin aging markers such as dermal structure, cell renewal, inflammatory response and oxidative stress mechanisms. Skin aging is a complex process involving numerous molecular mechanisms, and the use of DNA macroarray provides a global profile of hundreds of genes. A replicative senescence model of NHDF was used to obtain cell cultures presenting an expression profile representative of aged cells. Indeed, normal somatic cells have a limited capacity to divide in culture, and eventually enter a state of irreversible proliferative arrest, termed replicative senescence.[39] Previous studies using DNA array technology reported that, during replicative senescence, dermal fibroblasts presented a deregulation of genes involved in various skin cellular processes such as inflammatory response, cell cycle regulation and signal transduction.[40,41] This deregulation was also observed during skin aging.[42] Thus, using this model, not only could we compare the gene expression profiles of treated and untreated aged cells but we could also compare those of treated aged cells and normal cells. We could thus assess the ability of aged cells treated with poplar bud extract to recover an expression profile similar to that of normal cells.

The transcriptional effect of poplar bud extract on NHDF is presented in Table 4. Among the detected genes, the expression of four genes was significantly increased, while one was decreased by treatment with poplar bud extract. Poplar bud extract modulated the expression of catalase antioxidant enzyme gene, whose role, in combination with other enzymatic and nonenzymatic antioxidant systems, is to reduce reactive oxygen species and thus to maintain the oxidative balance in cells.[19] We

observed a significant increase in catalase gene expression in normal cells compared to untreated aged cells (+69%), confirming that antioxidant defenses decline during aging. This gene was strongly upregulated by poplar bud extract as treated aged cells presented a 2-fold expression increase compared to that found in normal cells (+130%). Therefore, poplar bud extract treatment allowed the aged cells not only to recover the expression level in normal cells but also to increase it more than 2-fold. These results are consistent with the antioxidant properties of poplar bud extract demonstrated previously and especially with a study reporting the stimulatory effect *in vivo* of some phenolic compounds identified in this extract (ferulic and *p*-coumaric acids) on the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase.[43] Altogether, these data suggest that, by its potential effect on catalase gene expression, this extract could attenuate the harmful effects of oxidative stress and reverse many of the events that contribute to skin aging.

Poplar bud extract also modulated the expression of CCL5 (chemokine (C−C motif) ligand 5) gene, a pro-inflammatory cytokine gene. This gene encodes a chemotactic cytokine involved in immunoregulatory and inflammatory processes. Also called RANTES (regulated on activation, normal T expressed and secreted), the pro-inflammatory CCL5 cytokine functions as a chemoattractant for T lymphocyte cells, eosinophils and basophils, and plays an active role in recruiting leukocytes into inflammatory sites,[44] thus contributing significantly to skin inflammatory disorders.[45] The expression of CCL5 gene was decreased in normal cells in comparison with untreated aged cells (−65%), suggesting that aged cells are more prone to inflammatory disorders. These results are consistent with a previous study, which reported the overexpression of CCL chemokine family genes in a replicative senescence model of human dermal fibroblasts.[41] Treatment of aged cells with poplar bud extract did not allow complete recovery of the expression level of CCL5 found in normal cells, but treated aged cells did moderately repress CCL5 gene expression in comparison with untreated aged cells (−39%). These results add to the existing data on the anti-inflammatory properties of *Populus* species[4,5] and poplar-type propolis.[15] These properties can be attributed to the presence of salicin, a phytotherapeutic precursor of salicylic acid known for its anti-inflammatory properties,[46] and to the presence of phenolic acids such as ferulic, caffeic and *p*-coumaric[47] and of some flavonoids such as pinocembrin.[48]

Furthermore, poplar bud extract significantly modulated the expression of KLF10, ZFP36L1 and E2F4, three genes involved in cell cycle regulation and turnover of the epidermis which are known to be downregulated in senescent human skin cells.[49] KLF10 and E2F4 belong respectively to the Krüppel-like and E2F family of transcription factors, and they play a role in cellular function, including cell proliferation, differentiation and survival.[50,51] KLF10 is also involved in immunoregulation of T lymphocyte cells.[52] ZFP36L1 encodes the epidermal growth factor response factor 1, which, when activated in response to epidermal growth factor (EGF), is involved in DNA synthesis and cell proliferation.[53] The expression level of KLF10, E2F4 and ZFP36L1 was higher in normal cells than in untreated aged cells (+57%, +110% and +75% respectively), confirming the deregulation of cell turnover in aged cells. Poplar bud extract presented a strong upregulation of these genes, with an expression level higher than that recorded in normal cells (+86%, +151% and +103% respectively). As poplar bud extract treatment of aged cells re-established the expression of normal cells, it might stimulate epidermal cell turnover, which is impaired in aging skin.

In conclusion, this study demonstrated the antioxidant properties of aqueous poplar bud (*Populus nigra*) extract thanks to its phenolic composition. Characterization of this extract led to the identification of caffeic and *p*-coumaric acids as its major antioxidant contributors as determined by ORAC assay. These compounds were found to represent together half of the total antioxidant activity of poplar bud extract. Caffeic acid also showed the highest antioxidant activity in cellular antioxidant activity (CAA) assay although this assay was weakly correlated with ORAC assay. Our results also indicate a potential beneficial effect of poplar bud extract on skin aging as it showed a strong modulation of transcription of genes involved in antioxidant defenses, inflammatory responses and cell renewal. Additional studies are needed to determine the bioavailability of poplar bud extract's phenolic compounds, the biological properties of these compounds and their metabolites *in vivo*, and to demonstrate their protective and curative skin antiaging effects, so they may be included in nutraceutical antiaging formulations.

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DISCLOSURE

Biolandes is involved in research/development and marketing/sales of the tested extract as ingredient for the nutraceutical industry. Therefore, Biolandes has a commercial interest in this publication. Polyphenols Biotech, the conducting laboratory, was paid by Biolandes to perform and report the scientific work that formed the basis of this publication. Polyphenols Biotech and Biolandes declare that the data in this publication represent a true and faithful representation of the work that was performed.

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