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Investigation of extracts from red and sugar maple buds as potential sources of antioxidant phytochemicals

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ABSTRACT

We report here for the first time, the results on the phenolic composition analysis of hot water and ethanol extracts of buds from sugar (*Acer saccharum*) and red maple (*Acer rubrum*), two dominant species of Laurentian forest of Quebec. The aim of the present study is to demonstrate the antioxidant capacity of these extracts while revealing their phenolic constituents. The extraction yields were determined for two types of buds based on the solvent used. Spectrometric methods were applied to quantify total phenols and different classes of polyphenols while the antioxidant capacity of the extracts was studied *in vitro* using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) test and Oxygen radical absorbance capacity (ORAC) assay. Higher extraction yields were obtained for red maple buds regardless of the solvent used. Hot water extraction yields were higher than ethanol extraction counterparts, for both maple buds studied. The spectrophotometric assays revealed that extracts from red maple buds contain higher concentration of total phenolics, flavonoids and anthocyanins while the concentrations of hydroxycinnamic acids and proanthocyanidins were higher in sugar maple bud extracts. Antioxidant capacity assays showed that, both sugar and red maple bud extracts presented real potential. Finally, strong relationships between the types and the amounts of phenolic compounds in maple buds and antioxidant capacity were determined. These results suggest that different valorisation fields for extracts from red and sugar maple buds could be anticipated.

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KEYWORDS: *Acer rubrum*, *Acer saccharum*, buds, extracts, phenolic contents, antioxidant capacity, PCA

INTRODUCTION

Sugar maple (*Acer saccharum* M., SM) and red maple (*Acer rubrum* L., RM), two dominant species of Laurentian forest of Quebec, are responsible for most of the red and orange autumnal coloration of Northeastern American forests. They are also widely recognised for their sap, which is used for maple syrup production. The various phenolic constituents of maple syrup have drawn a huge research attention due to their potential positive effects on human health. Traditional and anecdotal medicinal claims for other parts of the plant in Amerindian medicine incited the interest to study different red maple tissues. Though up until now, leaves and bark of these trees have been extensively studied [1-4], only one study was published so far dealing with the involvement of phenolic compounds in the bud dormancy breaking [5].

However, buds are the primary shoot-producers for dicotyledonous plants, and thus play a key role

in the growth and plant architecture. They contain important amount of meristems, the undifferentiated embryonic tissues where regrowth of new tissues takes place after winter dormancy [6]. Bud extracts are also a category of plant products well known and widely used not only in gemmotherapy but also in homeopathy and in phytotherapy [7]. Tree buds are generally rich in plant growth hormones, microelements, vitamins, enzymes, free amino acids, polyphenols, nucleic acids and other bioactive compounds that are often found only in trace amounts in differentiated parts of plants [8].

Among secondary plant metabolites, phenolic compounds represent potent antioxidants widely distributed in plant kingdom, particularly important in lignified tissues. They can be used as protective agents against oxidative damage of foods and biological systems [9]. However, finding a novel and natural resource, and green and efficient extraction methods which lead to safe and economical bioactive natural products still remains a real challenge [10]. The pruning of trees is a common practise performed in hardwood plantations, which would thus represent a potential source of branches containing buds, thus available for transformation and further use [11]. On the other hand, forest operations related to harvest also yield branches which are otherwise not forwarded to wood transformation industry. These branches contain buds which could be thus collected and forwarded into extraction plant [12]. In this context, the possibility of extraction of antioxidant phenolic compounds in significant amount from maple buds using environmentally friendly solvents and methods could lead to various valuable products.

To the best of our knowledge, this is the first study of sugar and red maple bud extracts obtained with green solvents, water and aqueous ethanol. The extracts were analyzed by spectrophotometric assays to quantify total phenols along with specific classes of phenolic constituents, while DPPH• radical scavenging assay and ORAC test were used to assess the *in vitro* antioxidant capacity.

MATERIALS AND METHODS

Plant materials

The dormant buds from sugar (*Acer saccharum*) and red maple (*Acer rubrum*) were harvested at the end of winter, from 10 to 24 March 2015. Voucher specimens (*Acer saccharum* Marsh, No. 176 and *Acer rubrum* L., No. 174) have been deposited at the herbarium of the Faculty of forestry, geomatics and geography (Faculté de foresterie géographie et géomatique) at Université Laval, Quebec City, Canada. The key morphological criteria described by Rouleau (1979) [13] were used to confirm their identities (Figure 1). Eight randomly selected vigorous trees (per species) were collected, pooled and mixed well before freeze-drying. Dried bud samples were then carefully crushed in a mortar to avoid the overheating and the powders were kept at -20 °C until extraction.

Extraction procedure

Maple buds (10 g) were extracted with 200 ml of solvent. The extraction with water was carried out using a water bath heated at 80 °C under reflux conditions for 1 hour. The extraction with ethanol (95%) was performed at room temperature by continuous shaking (230 rpm) for 24 h with an orbital shaker from Barnstead Lab-Line model 4633 (Melroso Park, IL, USA). For each extraction, the procedure was repeated twice at the same conditions to extract maximum of extractives.

The extracts were separated by filtration through Whatman No. 3 filter paper (Whatman International Ltd., UK) in a Buchner funnel. The recovered solvent (ethanol) was evaporated under vacuum at 50 ºC using a rotary evaporator (Rotavapor® model R-215) until dryness, while aqueous extracts were firstly pre-concentrated under vacuum evaporator using same conditions and then freeze-dried. The yields of extraction were expressed as a percentage of the initial oven dry plant material (w/w).

Spectrophotometric determination of phenolic contents

Total phenol content

The total phenol content of each sample was assessed according to the Folin-Ciocalteu's method, as described by Scalbert *et al*., (1989) [14]. Gallic acid (Sigma Chemical Co., St. Louis, MO, USA) was used as a standard for calibration curve. The total phenol content was expressed in gallic acid equivalents (mg. GAE/g dry extract).

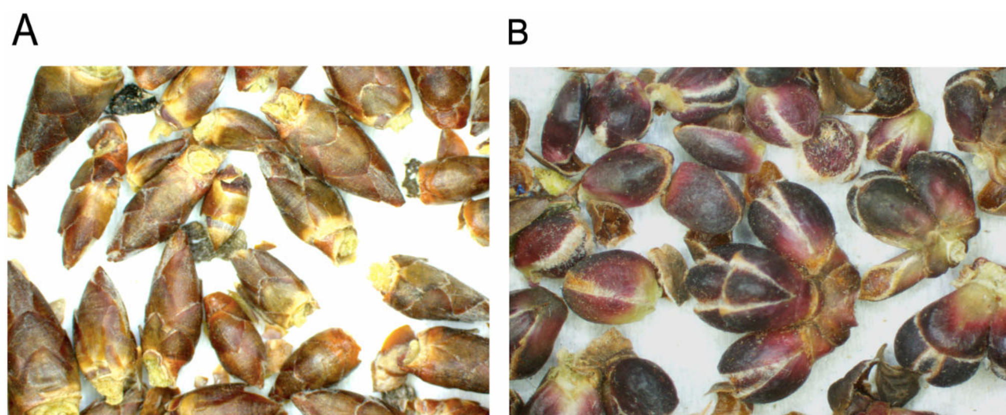

Figure 1. Morphology of maple buds. **A.** Sugar maple (*Acer saccharum* Marsh); **B.** Red maple (*Acer rubrum* L.).

Total flavonoid content

The total flavonoid content was determined according to the aluminum chloride method ($AlCl_3$) developed by Brighente *et al*., (2007) [15]. The total flavonoid content was calculated as quercetin equivalents (mg. QE/g dry extract) using a calibration curve obtained for quercetin hydrate standard (Sigma Chemical Co., St. Louis, MO, USA).

Hydroxycinnamic acids content

Determination of hydroxycinnamic acid content in maple bud extracts was performed according to the method used by St-Pierre *et al*., (2013) [16]. Chlorogenic acid (Sigma-chemical, St. Louis, MO, USA) as a calibration standard was used to express the hydroxycinnamic acid content (mg. CAE/g dry extract).

Determination of proanthocyanidins

Evaluation of proanthocyanidins was performed according to the procedure described by Wallace *et al*., (2010) [17] with slight modifications. Twenty microliters of samples (1 mg/mL) were diluted with 2.380 ml of methanol and then 100 µL of 4-dimethylaminocinnamaldehyde (DMACA) reagent (2% w/v in a methanolic sulfuric acid solution 6 N) were added. Protected from light, the mixture was incubated at room temperature for 30 min, and the absorbance of mixture was then recorded at 640 nm against a blank (methanol in place of DMACA reagent). The results of this assay were expressed in catechin equivalent (mg CE/g of dry extract).

Determination of total monomeric anthocyanin

Determination of anthocyanins in the bud extracts was conducted according to the pH differential method described by Lee *et al*., (2005) [18]. Total monomeric anthocyanins were determined using cyanidin 3-O-glucoside (Sigma Chemical Co., St. Louis, MO, USA) standard curve (mg C-3-gE/ 100 g of dry extract).

Antioxidant capacity evaluation

DPPH• free radical scavenging capacity test

The scavenging capacity of studied extracts for DPPH free-radical was assayed according to the protocol described by Li *et al*., (2009) [19]. The percentage of radical scavenging capacity (RSC) was expressed in terms of micromoles of Trolox equivalents per gram of dry extract (µmol TEq./g) using a calibration curve obtained for this standard.

Oxygen radical absorbance capacity assay (ORAC assay)

The capacity of maple bud extracts to slow down oxidation of a probe molecule (fluorescein) in the presence of peroxyl radical (AAPH) (Sigma Chemical Co., St. Louis, MO, USA) was assessed by the ORAC assay using the method described by Kasangana *et al*., (2015) [20]. The $ORAC_{FL}$ values of bud extracts were expressed in terms of micromoles of Trolox equivalent per gram of dry extract (µmol TEq./g dry extract).

For all antioxidant capacity evaluations, the commercial standardized French maritime pine bark extract Oligopin® was used for comparison.

Statistical analysis

Polyphenol assessments and antioxidant capacity determinations were expressed as means of three independent determinations ± standard deviation (SD). To assess the significance of the main effects of factors (nature of maple bud samples and type of extraction), the data sets were submitted to factorial analysis of variance (ANOVA), with the subsequent use of planned comparisons (contrast analysis), using the general linear model (*glm*) procedure of the SAS software package (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Extraction yields of maple bud samples

Extracts of maple buds were obtained with hot water and 95% aqueous ethanol. Both of these solvents are considered eco-friendly and non-toxic, and convenient to food and pharmaceutical processing [21]. The yields of extraction for all of these maple samples are presented in figure 2. Hot water extraction yields were consistently determined to be higher for all maple samples tested (45.3 ± 1.2 and 23.2 ± 0.2 for red maple and sugar maple buds, respectively) than those determined for ethanol extracts (37.5 ± 0.5 and 14.4 ± 0.2 for red maple and sugar maple buds, respectively). These findings are in agreement with those described in the literature [9] in which higher yields were obtained with hot water extraction. The higher polarity of the solvent (water versus ethanol) associated with a more elevated extraction temperature involved, could explain the increase in mass transfer rate resulting in higher extraction yields [21].

The planned contrast analysis indicated that, regardless of the solvent, extraction yields obtained from red maple buds were higher than those from sugar maple buds. These observations suggest potentially crucial differences in the chemical composition of red and sugar maple bud extracts. Consequently, according to the breadth of these variations, different applications could be considered for the buds of these two maple species.

It is noteworthy that very few buds of North American hardwoods have been chemically studied. Poplar buds [22], beech [23] and birch buds [24] have already been investigated, but data on their extraction yields have not been provided. Also, some studies have focused on other sugar maple and red maple tissues (wood, leaves, bark). For instance, Royer *et al.* [9] reported the hot water extraction yields from red maple whole branches, wood of branches, bark of branches, stem bark and whole stems ranging from 7 to 24% and from 4 to 12.5% for extraction yields with ethanol. In general, the values of extraction yields reported in the literature for various maple tree tissues were lower than those obtained for buds in this research. This could suggest that bud extracts will potentially provide high amount of bioactive compounds.

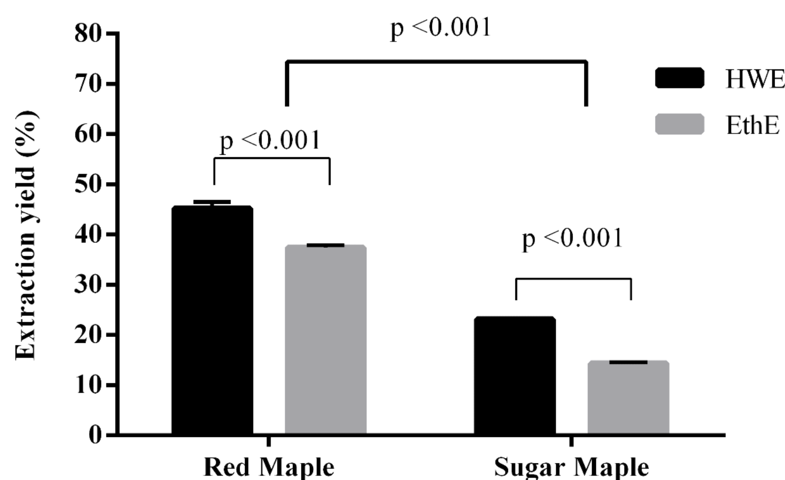

Figure 2. Extraction yields of maple bud samples.

Quantification of phenolic compounds in maple bud extracts

Among the renowned natural antioxidants occurring in plants (ascorbic acid, tocopherols, carotenoids etc.), phenolic compounds represent ubiquitous components of vascular plants. Phenolic acid derivatives, flavonoids, proanthocyanidins, anthocyanins, stilbenes etc. are some of main subgroups of phenolic compounds [25]. The interest for plant polyphenols is growing due to multiple potential applications associated with the bioactivity of these molecules. In this research, the contents of following classes of polyphenols in maple bud extracts, namely flavonoids, hydroxycinnamic acids, proanthocyanidins, and anthocyanins, along with total phenol content, were determined and the obtained results are summarized in table 1.

By comparing bud origins (red *vs*. sugar maple) and solvents used for extraction (hot water *vs*. ethanol), statistical analysis provides data on the influences of these extraction parameters on each class of phenolic compounds determined.

The results of the statistical analysis (in complementary data), indicate that hot water is a more efficient solvent for flavonoids than aqueous ethanol for red maple buds (10.1 ± 0.4 mg QE./g compared to 6.5 ± 0.3 mg QE./g) while somewhat higher flavonoid content was found in ethanol than in the water extract of sugar maple buds.

The total phenol content was found to be significantly higher in the red maple bud extracts than in sugar maple bud extracts for both extraction solvents. The total phenol contents of 458.4 ± 9.9 and 378.6 ± 1.1 mg GAE/g were determined for the extracts from red maple buds with hot water and ethanol 95%, respectively. On the other hand, only 280.4 ± 8.8 and 211.7 ± 9.6 mg GAE/g of total phenol contents were obtained with hot water and ethanol 95% for sugar maple buds. Consequently, the use of hot water as a solvent gave better results in terms of both extraction yields and total content of phenols irrespective of the nature of maple buds used.

Whatever the solvent used, the quantities of hydroxycinnamic acids and proanthocyanidins were determined to be consistently higher in sugar maple bud extracts than in the red maple counterpart. The nature of solvent showed very slight effect on the extraction of these groups of polyphenols.

The occurrence of precipitate in the aqueous ethanol extracts during the assessment of total anthocyanin content did not allow their complete evaluation. However, as the color of the extracts already indicated, the highest amount of anthocyanins was determined in red maple bud extract with hot water.

It has been shown in our previous study [9] that the stem bark extracts contained the highest amount

Table 1. Evaluation of total phenols and phenolic sub-class contents in sugar and red maple bud extracts.

Experiments	Factors		Variables				
	Solvents	Samples	TPC	TFC	THA	PAs	TMA
1	HW	RM	458.4 ± 9.9	10.1 ± 0.4	31.5 ± 1.6	33.1 ± 2.0	103.3 ± 9.8
2		SM	280.4 ± 8.8	5.2 ± 0.5	230.8 ± 14.2	125.1 ± 5.1	26.2 ± 3.6
3	EtOH 95	RM	378.6 ± 1.1	6.5 ± 0.3	49.9 ± 3.3	42.1 ± 1.2	75.3 ± 9.3
4		SM	211.7 ± 9.6	6.1 ± 0.3	226.2 ± 17.2	121.8 ± 7.9	nd

The reported results are means ± standard deviation (n = 3). Factorial analysis of the variance (ANOVA) was followed by planned orthogonal contrasts (statistical results shown in complementary data). The determinations were performed on hot water (HW) and 95% aqueous ethanol (EtOH 95) bud extracts of red maple (RM), and sugar maple (SM). Total phenol contents (TPC) were expressed in mg of gallic acid equivalents (GAE) per g of dry extracts; Total flavonoid contents (TFC) in term of mg of quercetin equivalents (QE) per g of dry extract; Total hydroxycinnamic acids (THA) in mg of chlorogenic acid equivalents (CAE) per g of dry extract; Proanthocyanidins (PAs) in mg of cathechin equivalents (CE) per g of dry extract; and Total monomeric anthocyanins (TMA) expressed in mg of cyanidin-3-glucoside equivalents per 100 g of dry extracts. nd (not determined).

of total phenols (494.3 ± 3.9 mg expressed in terms of tannic acid equivalent/g of dry extract) and the best proanthocyanidin content (30.1 ± 0.2 mg of cyanidin chloride equivalent/g of dry extract) was determined for bark ethanol extract. The same plant tissue extract was determined to have the highest total flavonoid content (14.1 ± 6.5 mg of quercetin equivalent/g of dry extract), which is somewhat higher than the contents determined for bud extracts studied in this research, while higher hydroxycinnamic acid content (53.9 ± 5.2 mg chlorogenic acid equivalent/g of dry extract) was determined in hot water than in ethanol extracts. In the present study, the most important finding in relation to hydroxycinnamic acid content is that it is consistently higher in sugar maple bud extracts, regardless of the solvent used for extraction. The use of different standards for the expression of some of the results, and considering that we favoured the spectrometric methods appropriate for the particularity of the plant material studied, make the comparisons with literature data difficult. The results obtained in this study remain, however, in the same order of magnitude as those reported previously for other maple tissue extracts.

Finally, although anthocyanins have already been highlighted in the leaves of maple species, this, to the best of our knowledge, is the first report on their quantification in maple buds. We have been using the AOAC Official pH differential Method (2005.02) for total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines. Anthocyanins represent an important group of water-soluble pigments in plants, responsible for the red, purple, and blue coloration, primarily of flowers, fruits and leaves of angiosperms [26]. The concentrations of anthocyanins in many foods and beverages have been estimated, and high concentrations are found mainly in fresh berries like cranberry (140 ± 28.5 mg/100 g of fresh weight), in vegetables such as red cabbage (322 ± 40.8) [27] and in red wines (240-350 mg/litre) [28]. The value of 100 mg/100 g of dry weight obtained for the red maple buds is lower than those reported for fruits and vegetables. However, the red maple bud anthocyanins are expected to have specific structures, which will be the basis for extensive chemotaxonomic studies of anthocyanins in maples from Aceraceae family.

Antioxidant activity of maple bud extracts

The interest in natural antioxidant was stimulated mainly by epidemiological studies indicating a relationship between the intake of foods rich in antioxidant and the incidence of several chronic diseases such as cardiovascular, diabetes, and cancer [29]. Thus, many *in vitro* assays have been developed to evaluate the antioxidant capacities of natural products. Two chemical reaction mechanisms are usually described to be involved in majority of these assays: Hydrogen atom transfer (HAT) and single electron transfer (SET) patterns. Thus, some authors recommend the use of at least two tests involving different mechanisms to investigate and understand the antioxidant potential of complex materials. We have therefore selected two tests, the first one based on a predominantly SET mechanism, which is a radical scavenging capacity assay implicating the reduction of a stable DPPH• radical and the measurement of the disappearance of its color in the presence of an antioxidant (phenolic constituents of the studied extracts). The second test, ORAC, measures the ability of an antioxidant to inhibit fluorescence decay of a probe, such as fluorescein from its reaction with the peroxyl radical (AAPH) by hydrogen donation [30]. The results of antioxidant activity tests are summarized in table 2.

Once again, the effects of extraction parameters on antioxidant capacity were revealed. The DPPH• radical scavenging capacity displayed no significant difference between hot water and ethanol bud extracts regardless of the maple species. Nevertheless, red maple bud extracts were determined to have more than 3 times higher DPPH antioxidant activities (3762 ± 476 and 3325 ± 129 µmoles of TE/g, for hot water and ethanol red maple bud extracts, respectively) than the sugar maple bud extracts.

Interestingly, in contrast to DPPH assay, the antioxidant activity tested by ORAC assay was determined to be significantly higher for sugar maple than for red maple bud extracts. However, the differences found for these samples were not as important as it was with the case for DPPH test. Statistical analysis (in complementary data) demonstrated that the ORAC values determined for hot water extracts of red maple were slightly higher than those determined for their ethanolic

counterparts (4305 ± 55 *vs.* 3740 ± 44 µmoles of TE/g), while no significant effect of solvent was noted for sugar maple bud extracts.

The DPPH• radical scavenging and ORAC assay were also performed on Oligopin®, a standardized hot water extract of maritime pine bark used as positive reference. The following values were determined for Oligopin®: 1930 ± 101 µmoles of TE/g for DPPH• radical and 14348 ± 92 µmoles of TE/g for ORAC. Comparing these results to those obtained for red maple bud extracts it can be noted that red maple bud extracts have radical scavenging capacity superior to Oligopin® when tested by DPPH•, but show inferior potential when evaluated by ORAC assay. Reported to be a polyphenol-rich extract, Oligopin® demonstrated higher amounts of all studied classes of polyphenols especially so for total phenolic content (572.9 ± 12.1 GAE. mg/g), total hydroxycinnamic acids (335.5 ± 3.7 CAE. mg/g) and proanthocyanidins (104.9 ± 9.6 CE. mg/g) than any of the maple bud extracts studied here [9].

Relationship between phenolic contents and antioxidant test

The determinations of phenolic contents, and the antioxidant capacity assessments were used as active variables in the derivation of principal component analysis (PCA). The objective of this analysis was to specify the relationship between the variables of the phenolic contents and the antioxidant capacity determinations (Figure 3). The Pearson correlations shown in table 3 were furthermore established between these variables in order to better define significance of these relationships.

The results obtained from these analyses demonstrate that DPPH radical scavenging activity presented significant positive correlation with total phenolic content and total flavonoids contents, but significant negative correlation with proanthocyanidins and total hydroxycinnamic acids. On the other hand, ORAC activity results showed a significant positive correlation with hydroxycinnamic acids and a negative correlation with total phenolic content.

Table 2. Antioxidant capacity of maple bud extracts as determined by DPPH and ORAC assays.

Experiments	Factors		Variables	
	Solvents	Samples	ORAC	DPPH•
1	HW	RM	4305 ± 55	3762 ± 476
2		SM	5941 ± 206	867 ± 52
3	EtOH 95	RM	3740 ± 44	3325 ± 129
4		SM	5981 ± 107	968 ± 155
5	OLIGOPIN®		14348 ± 92	1930 ± 101

Table 3. Pearson correlation coefficients between variables (quantities of each class of phenolic compounds and the antioxidant tests).

	Pearson correlation coefficients			
Variables	TPC	TFC	THA	PAs
ORAC	-0.8737 **(p = 0.0229)***	-0.67539 **(p = 0.1410)**	0.96737 **(p = 0.0016)****	0.9584 **(p = 0.0026)****
DPPH	0.9570 **(p = 0.0027)****	0.83867 **(p = 0.0369)***	-0.9991 **(p < 0.0001)****	-0.9946 **(p < 0.0001)****

TPC: Total phenolic content; TFC: Total flavonoid content; PAs: Proanthocyanins; THA: Total hydroxycinnamic acid. *indicates statistically significant test at $P < 0.05$; **indicates statistically highly significant test at $P < 0.01$.

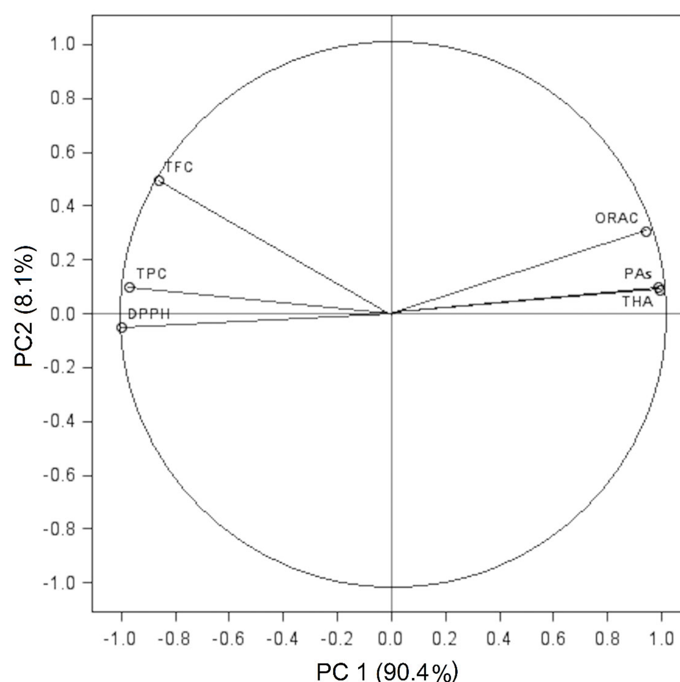

Figure 3. The correlation circle of phenolic compound classes and the antioxidant capacity assays.
TPC: Total phenolic content; TFC: Total flavonoid content; PAs: Proanthocyanins; THA: Total hydroxycinnamic acid.

These findings seem to corroborate with those described previously which also demonstrated that the effectiveness of *in vitro* antioxidant test was related to the nature of phenolic compounds. It has been demonstrated [31] that the important physicochemical parameters such as the bond dissociation energy (BDE) of the phenolic O-H and the ionization potential (IP) of the compound might favour electron or hydrogen transfer mechanisms in the phenolic compounds. For this reason, phenolic acids [32] and proanthocyanidins [33] were often suggested to be strongly correlated to the ORAC assay, which is based on hydrogen atom transfer. Thus, this would explain the high ORAC values obtained for the sugar maple bud extracts in the present study (having high total hydroxycinnamic acid contents), whereas red maple bud extracts rather exhibited strong DPPH• radical scavenging capacity.

CONCLUSION

We present for the first time, the results of phenolic composition and antioxidant capacity study of sugar and red maple bud extracts obtained with hot water and aqueous ethanol.

Higher yields of extraction were determined for red maple buds regardless of the solvent. The spectrophotometric determination of the phenolic contents revealed the highest contents of total phenols, flavonoids and anthocyanins in red maple extracts, while sugar maple bud extracts were determined to have higher concentrations of hydroxycinnamic acids and proanthocyanidins. The nature of the solvent used for extraction seems to be of importance for better targeting phenolic compounds of interest from maple buds, as higher yields and contents were determined for water extracts. The antioxidant capacity assays showed that, both sugar and red maple buds extracted by hot water present really good antioxidant potential.

These major findings bring important elements of decision in terms of choice of maple buds according to species and of green solvent to be used for extraction of targeted phenolic compound class. The *in vitro* validation of biological properties and the identification of bioactive phenolic constituents of the studied extracts make part of our ongoing research. The results, once obtained, will confirm the applicability of maple bud extracts

and/or their active ingredients for the design of new innovative non-wood forest products (NWFP).

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COMPLEMENTARY DATA (Statistical analysis results)

A. Factorial analysis of the variance (ANOVA) for spectrophotometric evaluation of phenolic content.

Variables	Source of variation	p value
TPC	**Maple buds**	**< 0.001****
	Solvents	**0.002****
	Maple buds * Solvents	*0.433* ns
TFC	Maple buds	< 0.001**
	Solvents	0.001**
	Maple buds * Solvents	**< 0.001****
THA	**Maple buds**	**< 0.001****
	Solvents	0.189 ns
	Maple buds * Solvents	0.280 ns
PAs	**Maple buds**	**< 0.001****
	Solvents	**0.033***
	Maple buds * Solvents	0.063 ns

B. Factorial analysis of the variance (ANOVA) for antioxidant capacity assays.

Variables	Source of variation	p value
ORAC	Sample	< 0.001**
	Solvent	0.010**
	Sample * Solvents	**0.002****
DPPH	**Sample**	**< 0.001****
	Solvent	0.193 ns
	Sample * Solvents	*0.0560* ns

*Indicates statistically significant test at $P < 0.05$.
**Indicates statistically highly significant test at $P < 0.01$.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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