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Identification of high-value bioactive constituents in Northern European willow varieties: *S.X. Dasyclados, Endeavour, Cheviot, Tora, Resolution, S. Purpurea, Terranova, endurance*

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ABSTRACT

Willow trees have maintained their place in medicine for many years as they are herbal source of various cures. Willow varieties are considered to contain a wide range of anti-inflammatory and anti-bacterial species such as salicylates and flavonoids. The current work is centred on the presence of high bioactive pharmaceutical constituents other than salicin such as flavan-3-ol catechin, salicortin, and other complex compounds, which contribute to the total medical value of willow extracts. To evaluate the distribution of these bioactives, bark, and wood fractions of 8 different willow varieties (S.*X. Dasyclados, Endeavour, Cheviot, Tora, Resolution, S. Purpurea, Terranova, Endurance*) were extracted using dispersive solid phase extraction and then analysed using LC-MS comprising a quadrupole time-of-flight spectrometer. Indeed, various high value constituents such as salicortin, catechin, triandrin, acacetin-5-O-xyloside, picein, apigenin-7-O-glucoside, vitexin-2-rhamnoside, luteolin-7-glucoside, catechin gallate, and kaempferol as well as giberellic and 5-methoxysalicylic acid were detected in bark and wood fractions of the willow varieties 80:20 ethanol/water extracts.

Introduction

Finding alternative energy resources has been always an ultimate goal for governments, as the world seeks to supplement fossil fuels, reduce CO_2 emissions, and improve the national economy. This renders biomass crops, one of which is willow, one of the key biomass resources for bioenergy. The full-scale planting of willow for bioenergy dates to early 1990s, where most plantation activities were held by Agrobransle AB and SL bioenergy companies owned by the Federation of the Swedish Farmers Co-ops (Larsson and Rosenqvist, 1996). The framework of this policy was implemented by imposing higher environmental and energy taxes on fossil fuels while exempting biofuels and subsiding set-aside lands for planting willow. The subsidies were near 1200 €/ha, plus 480 €/ha for fencing (Anon, 1990). Moreover, the White Paper of the European Union expected a growth in bioenergy share from 3 to 8.5% by 2010, half of which is sourced from energy crops (European Commission, 1997).

Historically, the value of willow has been always seen as an energy crop from an industrial perspective, without attempting to valorise the bioactives present in its fractions. Additional assessment of the composition of different willow fractions, such as bark, leaves, and pulp is therefore warranted. It is reported in literature that bark and leaf fractions contain polyphenolics and a large fraction of compounds which are bioactive and possess pharmaceutical value, whilst the pulp fraction is used to produce biomaterials (e.g., packaging materials) (Pisano, Gottumukkala, Hayes, and Leahy, 2021).

The pharmaceutical value of willow lies in its bark part, either in the young branches or in the one-year-old twigs fragments (European Pharmacopoeia 5.2, 2005). Notably, bark extract containing salicylic acid as a non-steroidal anti-inflammatory drug is evaluated as a medication to cure fever and inflammation. Even though salicin, the precursor of salicylic acid, constitutes a major fraction of bark extracts, other ingredients such as flavonoids (flavan-3-ol catechin, gallocatechin, epicatechin) and glycosylated flavonoids account for 20% of willow bark (Förster et al., 2021; Shao, 1991). Several higher molecular mass salicylates including salicin, salicortin, salicylic acid, and tremulacin

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further highlights willow bark extract as medicinal plant for hindering infections (EMA, 2017; Fötsch and Pfeifer, 1989; Krantz, Berger, and Hiatt, 2010).

Acknowledging the fact that salicylic acid derivatives such as acetyl salicylic acid (ASA) are well-known non-steroidal anti-inflammatory drugs, it is obvious that such statements about the effectiveness of willow bark against infections will hardly do justice to the contribution of various compounds that exist along with salicin. Noteworthy, clinical research performed by Mayer et al. shed light on the possible role of constituents other than salicin (Mahdi, 2010). In addition, the main question as to whether the attractive overall activity of willow bark is exclusive to salicin has been answered through more clinical studies run by Lardos et al (Lardos et al., 2004). It was found that introducing different salicin dosages did not contribute to the efficacy of the extracts, which agrees with findings by Mahdi, JG. et al. (Mahdi, 2010).

The chemo-preventive role of salicin and other constituents including flavonoids, salicylalcohol derivatives, and proanthocyanidins, suggests willow bark extractives as attractive substances having phytopharmaceutical value (Hostanska, Jürgenliemk, Abel, Nahrstedt, and Saller, 2007). Outstanding findings have been achieved in this area; a conclusive proof of its efficacy in inhibiting cancer cells in human lungs and colon was obtained by experimental work using flow cytometry and by assessing light scattering characteristics (Hostanska et al., 2007). Anti-proliferative activity with 50% maximal growth inhibitory concentration was observed at concentrations between 33.3 and 103.3 μg/ml for flavonoids and proanthocyanidins, respectively, and 50.0-243.0 μg/ml for salicylic alcohol derivatives (Hostanska et al., 2007). Moreover, decoctions of willow bark and leaves with high content of salicylates for fighting rheumatism suggests willow bark as a herbal source of antiseptics (Vane, 2000).

Acknowledging the attractive pharmaceutical value of other polyphenolic groups that adds to the analgesic potency in bark extract, the importance of willow is further expanded by shifting from using salicin only, to exploiting other bioactive compounds. Compared to salicylates, less attention has been given to flavonoids despite their attractive role as antioxidants and as cancer treatment. In addition, a large variation of the distribution of phenolic glycosides and the total polyphenolic groups exists in willow bark due to different genotypes (Nahrstedt, Schmidt, Jäggi, Metz, and Khayyal, 2007), bringing about a whole new set of new challenges. Therefore, we investigated the presence of other polyphenolics rather than salicin in 8 different willow varieties: *S.X. Dasyclados, Endeavour, Cheviot, Tora, Resolution, S. Purpurea, Terranova, Endurance*. The study also provides an extraction protocol using ethanol, which can be easily recovered for reuse. The reason for the compositional characterisation of the willow varieties is to demonstrate the presence of several constituents, which might contribute to the overall anti-inflammatory effect of the plant extracts. Further studies will be conducted on the effect of soil quality in relation to the concentration of polyphenolics quantified in several willow varieties.

Materials and methods

In this work, ethanol (99%+, extra pure absolute, Fisher Chemical E/0600DF/17) and formic acid (99.0+%, Optima ™ LC/MS Grade, Fisher Chemical ™ A117-2AMP) were purchased from Fisher Scientific. Acetonitrile (hyper grade for LC-MS LiChrosolv®) was purchased from Merck Life Science Limited. Throughout the entire work, ultrapure water was used of 18 M_{Ω} ELGA purification system. The Q-discs G1, C9, S1 were purchased from CEM Technology Ltd.

Planting material

A horizon scan of different willow breeding programs was conducted to choose a number of willow genotypes and varieties which would be likely to produce levels of compounds interesting from a medicinal perspective. These selections not only focused on the progeny of certain crossings but of the parent material also. A total of 39 willows were therefore chosen for the trials, the justification for which was based not only on the compound production potential but also on their ability to grow fast with reasonable yields and with good form to enable mechanical harvest (straight up from stool and little in the way of side branching). The final selection included a number of current and old commercial varieties but also near market lines and some interesting crossing selections.

Planting design and location

The trial design and randomization were such that each of three blocks comprised 39 randomized plots. 40 willow cuttings were planted in each of the plots in a planting arrangement similar to that performed by a mechanical step-planter. The plots consisted of 2 double rows of ten plants with 0.75m between the willows in each double row and 1.5m between the two double rows. Within the rows, the willows were planted 0.60m from each other (a planting density therefore of 16,600 plants ha^{-1}). Each plot therefore measured 3m by 5.4m. The blocks measured 19.8m by 61.5m. The trials were planted at three locations which were The Agrifood and Biosciences Institute, Loughgall. Co. Armagh, N. Ireland (54.40856582671539, -6.597683949044219), The University of Limerick planting site near Claremorris, Co Mayo, Republic of Ireland (53.72851943138723, -8.954169512565535) and at Route de Vaulx, 62128, Noreuil, France (50.16301968905896, 2.9246611344383338).

Harvesting

A portion of each plot was harvested in February 2022 as per best practice guideline (Barry Caslin, 2015). To do this, 12 stools were fully harvested, strimmed at normal harvest stool height, from one end of the plot and as such leaving a row to 'guard' the willows for a second harvest to produce two-year-old material in Feb 2023. All the biomass material from each individual stool was removed and subsequently weighed, measured (average lengths and diameters) and extent of any branching noted.

Debarking

A manual debarking system was assembled to cleanly strip bark from a number of selected rods from each plot (approx. 100g) for onward analysis. This gave triplicate samples of bark of each variety for onward analysis. Some genotypes/varieties had more vigorous growth and these were used for further bulk bark preparation (10kg-15kg) for larger extraction work and for clean wood supply for materials research.

Extraction

The extraction process was executed by using EDGE®, automated solvent extraction system manufactured by CEM Technology Ltd. The instrument is equipped with cups which are furnished with disc filters (S1, G1, C9) at the bottom. The discs are cellulose filter discs that allow the filtration of the liquid extract for further analysis. Prior to sample preparation, an S1 Q-disc was preassembled whereby G1 Q-disc was sandwiched by two C9 Q-discs and then was located at the bottom of the Q-cup. Afterwards, about 0.5 grams of debarked and chopped (10-40mm particle size) bark or wood feedstock was placed over the S1 Q-disc.

Since salicin is hydrophilic, the extraction protocol employed high organic content 80:20 ethanol/water solvent system for 20 minutes hold time at 85°C to target other constituents than salicin (Table 1). The above-mentioned extraction protocol is elucidated in Fig. 1.

Chromatography

The LC-MS system used was Agilent 1260 HPLC Infinity + Agilent

Table 1
Extraction protocol cycle.

Cycle	Top add (ml)	Bottom add (ml)	Rinse (ml)	Temp. (°C)	Hold time (mm:ss)
1	20	10	10	85	20:00

6530 fitted with a DAD (diode array detector) detector and a QToF (quadrupole time-of-flight) mass spectrometer. The chromatographic separation was performed using a Phenomenex Luna C18 column 5um particle size (150 mm x 4.6mm). The separation protocol was developed based on gradient conditions (Table 2) using a mobile phase of acetonitrile and water with 0.1%vol. formic acid. The injection volume was set at 5 μL and the column temperature at 35°C. The binary pump was fixed at a flow rate of 1 ml/min. The UV spectra were collected at 267 nm for each run. All the sample samples were then analysed with the QtOF under negative and positive electrospray ionization (+/- ESI), with a m/z (mass-to-charge ratio) range of 50-3200. The collision voltage was set to 0, to isolate the main ions, without further complex fragmentation.

Results

The analysis of extracts of willow varieties was achieved using liquid chromatography equipped with a DAD detector and a quadrupole time-of-flight (QToF) mass spectroscopy. The chromatogram from the DAD detector shows the peaks of the constituents possessing UV absorptions at different wavelengths and retention times. Using the MassHunter™ software, the corresponding MS spectra was extracted from each DAD peak, yielding the relative abundance of mass to charge fragments (m/z) of the related species. For instance, the UV chromatogram of the bark fraction of *S.X. Dasyclados* variety is shown in Fig. 2.

The identification of the high-value constituents in the 8 selected willow varieties was based on recognizing mass-to-charge values (m/z) of the fragments and on the comparison with the mass spectra online database MassBank. For instance, the extracted mass spectra of the peak at retention time 5.753 min (Fig. 2) showed a fragment of 289.0732 m/z with the highest abundance using negative ionization (Fig. 3). Using MassBank, this charged fragment corresponds to deprotonated catechin [M-H]- and thereby the peak at 5.753 min was confirmed as catechin.

Table 3 exhibits the present constituents in wood and bark fractions of S.X. Dasyclados, Endeavour, Cheviot, Tora, and Resolution varieties using the negative ionization mode (-ESI) of the QToF. Therefore, the tabulated values are the deprotonated molecules denoted [M-H]- along with the corresponding adduct.

Table 4 elucidates the high-value constituents identified in wood and bark fractions of *S.X. Dasyclados, Endeavour, Cheviot, Tora, and Resolution* varieties using the positive ionization of QToF MS. The listed constituents mark [M+H] + protonated compounds with the absence of adducts. It was noticed that sodium adducts appeared along with salicin and rosin in *Tora* bark and *S.X. Dasyclados* bark, respectively.

The identified high-value constituents in the bark fraction of *Terranova* and *Endurance* varieties under both ionization modes (+/-ESI) are tabulated in Table 5. Ionized salicin fragments in *Terranova* bark and *Endurance* bark generated formate adduct of (-ESI) 311.1121 m/z and (-ESI) 311.1102 m/z, respectively. Whilst sodium adducts corresponded to salicin constituent in *Terranova* bark and *Endurance* bark under positive ionization of (+ESI) 309.0929 m/z and (+ESI) 309.1098 m/z, respectively. The formate adducts clearly appeared under negative ionization (-ESI) for *S. Purpurea* bark with salicin, salicortin, and 5-methoxysalicylic acid but no presence of them was noted under positive ionisation mode (+ESI).

Discussion

The choice of the extraction solvent was driven by the aim of this work to target bioactive compounds with anti-inflammatory properties rather than salicin in willow bark. The high solubility of the target flavonoids molecules in organic solvents relative to that in water played a key role in selecting ethanol (Ferreira and Pinho, 2012).

In fact, salicin possess a solubility of 288.608 10^5_{xA}cal in water while only 62.691 10^5_{xA}cal in ethanol at 25°C (Huang et al., 2020). Nevertheless, our results showed that salicin was still identified in the bark extracts of most tested varieties. In fact, it was reported elsewhere that

Table 2
Chromatographic separation gradient protocol.

Time	%A (Water)	%B (Acetonitrile)
0	95	5
3	50	50
5	10	90
12	10	90
18	50	50
20	95	5

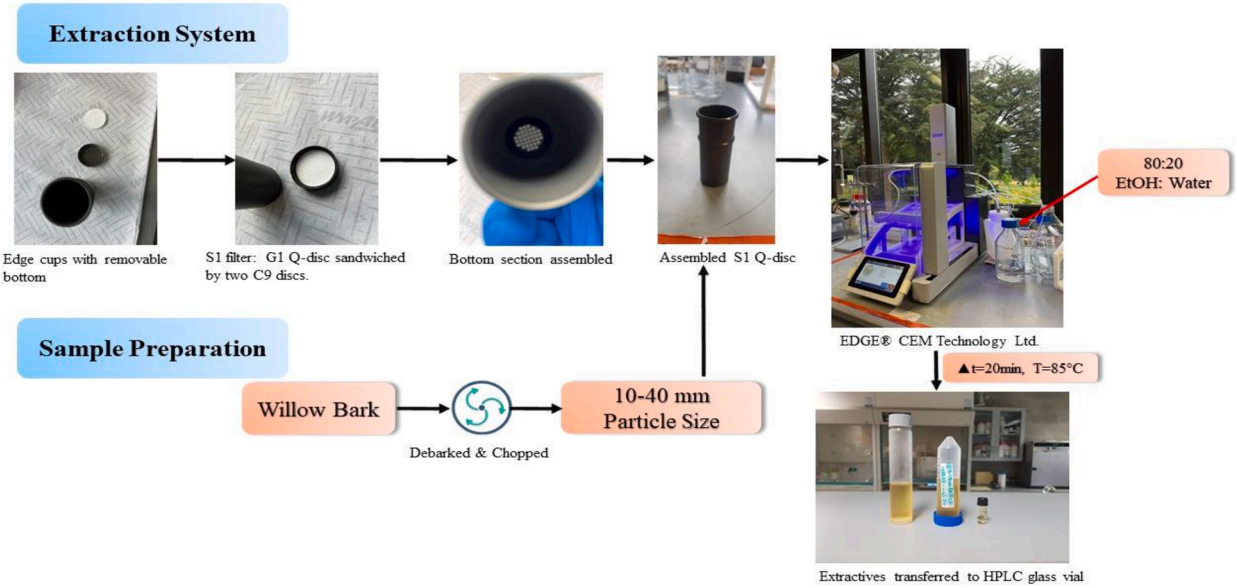

Fig. 1. Willow bark sample preparation and extraction procedure block diagram.

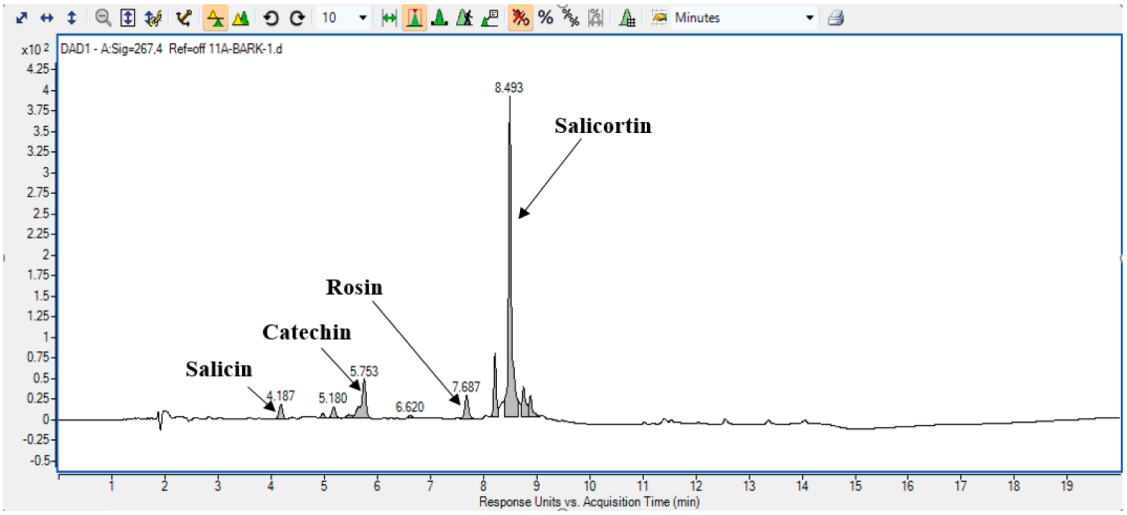

Fig. 2. S.X. Dasyclados bark fraction UV chromatogram at the following conditions: injection volume 5 μL; 1 ml/min flow rate; column temperature 35°C; run time 20 minute.

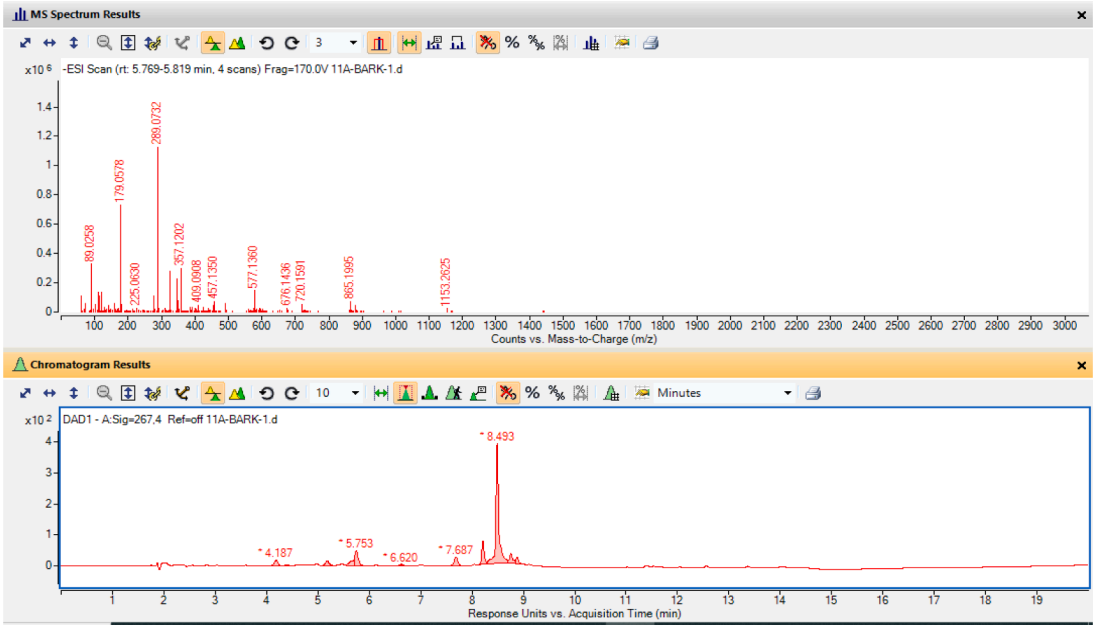

Fig. 3. Extracted mass fragments from catechin UV peak at retention time 5.753 min: 289.0732 m/z corresponding to [M-H].

ethanol-miscible compounds contribute to the usefulness of willow bark extracts. For instance, some studies have demonstrated the ability of ethanolic crude extracts to inhibit cell inflammation by hindering immunoactivity and COX-2-mediated PGE2 release, proving the anti-inflammatory effect of the organic solvent extracts of *Salix cortex* (willow bark) (Bonaterra et al., 2010). Various experiments using in-vitro and in-vivo tests on willow bark extracts also assessed different solvent mixtures. Recently, the hydro-alcoholic (water/methanol 70:30) extracts of *Salix babylonica* were examined in-vitro against drug-resistant bacteria such as *Aeromonas hydrophila, Listonella Anguillarum, Edwarsiella Tarda* as well as non-drug resistant bacteria such as *Streptococcus Iniae,* providing promising results in terms of anti-bacterial action (Rangel-López et al., 2020). Regarding in-vivo experiments, willow crude extracts from different solvents (toluene, ethyl acetate, butanol, ethanol) were introduced to rats for treating arthritis. It was found that crude ethanolic extracts have as same anti-inflammatory effect performance as acetylsalicylic acid, which is the active ingredient of Aspirin (Nahrstedt et al., 2007). However, the question of which compounds were responsible for this action remains unanswered (Bonaterra et al., 2010). In addition, other experiments have also proved that water extracts are less effective in terms of anti-inflammatory activity than extracts obtained from organic solvents, such as ethanol and methanol (Antoniadou et al., 2021).

Salicortin was identified in the 80:20 EtOH: H_2O (ethanol: water) bark extracts of the Salix varieties, including *Salix X. Dasyclados, Endeavour, Cheviot, Tora, Salix Purpurea, Terranova* and *Endurance*. For example, in *S.X. Dasyclados*, a main fragment corresponding to the deprotonated molecular ion of salicortin and its formate adduct were observed at 423.1312 m/z and 469.1364 m/z, respectively (Table 3). Similar fragmentation patterns were observed in the bark fraction of the other varieties.

Salicortin is envisaged responsible for the anti-inflammatory activity of willow bark extract using 80:20 aqueous ethanol. This is demonstrated by its ability to reduce the IKKαβ pathway, which is primarily

Table 3
Identification of high-value constituents in bark and wood of S.X. Dasyclados, Endeavour, Cheviot, Tora, and Resolution varieties under negative ionization mode (-ESI).

Constituents	$[M-H]^-$ (m/z) and other fragments *S.X. Dasyclados* Bark	*S.X. Dasyclados* Wood	*Endeavour*Bark	*Endeavour*Wood	*Cheviot*Bark	*Cheviot*Wood	*Tora*Bark	*Tora*Wood	*Resolution Bark*	*Resolution Wood*
Catechin $C_{15}H_{13}O_6$ 290.26 g. mol^{-1}	X *289.0732 [M-H]-; 179.0578*	X *289.0744 [M-H]-; 179.0579*	X *289.0747 [M-H]-; 179.0584*	X *289.0779 [M-H]-; 179.0584*	-	X *289.0806 [M-H]-; 179.0608*	-	-	X *289.0770 [M-H]-; 179.0601*	X *289.0797 [M-H]; 179.0618*
Salicin $C_{13}H_{17}O_7$ 286.28 g. mol^{-1}	X *285.0998 [M-H]-; 331.1050 formate adduct*	-	X *285.1000 [M-H]-; 331.1055 formate adduct*	-	X *285.1043 [M-H]-; 331.1102 formate adduct*	-	X *285.1054 [M-H]-; 331.1112 formate adduct*	-	-	-
Salicortin $C_{20}H_{23}O_{10}$ 424.4 $g.mol^{-1}$	X *423.1312 [M-H]-; 469.1364 formate adduct*	-	X *423.1338 [M-H]; 469.1386 formate adduct*	-	X *423.1378 [M-H]-; 469.1431 formate adduct*	-	X *423.1394 [M-H]-; 469.1450 formate adduct*	X *423.1429 [M-H]; 469.1490 formate adduct*	-	-
Triandrin $C_{15}H_{19}O_7$ 312.31 g. mol^{-1}	-	X *311.2045[M-H]-*	-	X *311.2073 [M-H]-*		X *311.2061 [M-H]-*		X *311.2088 [M-H]-*		X *311.2095 [M-H]-*
Acacetin-5-O-xyloside $C_{21}H_{19}O_{19}$ 416.1 $g.mol^{-1}$	-	-	X *415.2006 [M-H]*	-	X *415.2042 [M-H]-*	-	X *415.2042 [M-H]-*	X *415.2100 [M-H]-*	-	-
Catechin (gallic acid complex) $C_{15}H_{13}O_6$ 290.26 g. mol^{-1}	-	-	-	-	X *289.0780 [M-H]-, 179.0608, 425.1737, 379.1677*	-	X *289.0793 [M-H]; 179.0615. 357.1274, 447.1602*	-	-	-
Luteolin-7-glucoside $C_{21}H_{20}O_{11}$ 448.10 g. mol^{-1}	-	-	-	-	-	-	X *447.1602 [M-H]*	-	-	-
Apigenin-7-O-glucoside $C_{21}H_{19}O_{10}$ 432.10 g. mol^{-1}	-	-	-	-	-	-	-	-	X *431.1978 [M-H]-*	-
Vitexin-2-rhamnoside $C_{27}H_{29}O_{14}$ 578.16 g. mol^{-1}	-	-	-	-	-	-	-	-	X *577.1427 [M-H]-*	-
Luteolin-7-glucoside $C_{21}H_{20}O_{11}$ 448.10 g. mol^{-1}	-	-	-	-	-	-	-	-	X *447.1567 [M-H]*	-
Catechin gallate $C_{22}H_{17}O_{10}$ 442.37 g. mol^{-1}	-	-	-	-	-	-	-	-	-	X *441.2071 [M-H]-*
Kaempferol $C_{15}H_9O_6$ 286.23 g. mol^{-1}	-	-	-	-	-	-	-	-	-	X *571.14632 [M-H]-*

Table 4
Identification of high-value constituents in bark and wood of S.X. Dasyclados, Endeavour, Cheviot, Tora, and Resolution varieties under positive ionization mode (+ESI).

Constituents	$[M-H]^+$ (m/z) and other fragments									
	S.X. Dasyclados Bark	*S.X. Dasyclados* Wood	*EndeavourBark*	*EndeavourWood*	*CheviotBark*	*CheviotWood*	*ToraBark*	*ToraWood*	*Resolution Bark*	*Resolution Wood*
Catechin	X	X	X	X	X	X	X	X	X	X
$C_{15}H_{13}O_6$	*291.0865*	*291.0858 [M+H]-*	*291.0867*	*291.0885*	*291.0872*	*291.0925*	*291.0877*	*291.0933*	*291.0868*	*291.0905*
290.26 $g.mol^{-1}$	*[M+H]+*		*[M+H]+*	*[M+H]+*	*[M+H]+*	*[M+H]+*	*[M+H]+*	*[M+H]+*	*[M+H]+*	*[M+H]+*
Salicin	-	-	-	-	-	-	X	-	-	-
$C_{13}H_{17}O_7$							*309.0962*			
286.28 $g.mol^{-1}$							*[M+Na]+*			
Rosin	X	-	-	-	-	-	-	-	-	-
$C_{15}H_{20}O_6$	*321.0607*									
296.31 $g.mol^{-1}$	*[M+Na]+*									
Picein	-	-	-	-	-	-	X	-	-	-
$C_{14}H_{19}O_7$							*299.1111*			
298.291 $g.mol^{-1}$							*[M+H]+*			
Vitexin-2-rhamnoside	-	-	-	-	-	-	-	-	-	X
$C_{27}H_{29}O_{14}$										*579.1580*
578.16 $g.mol^{-1}$										*[M+H]+*

caused by the inflammatory response of conditions such as obesity (Harbilas et al., 2013). Other studies have also proved the effectiveness of salicortin in reducing the expression of the mediators that cause inflammation conditions in macrophages immune cells, which are responsible for treating auto-immune diseases and infections (Kwon et al., 2014).

Another bioactive compound identified in all the *Salix* varieties was catechin. Catechin fragmentation patterns were evident in both negative and positive ionization modes. Typical fragments of 289.0762 m/z in negative mode (Table 3) and 291.0865 m/z in positive mode (Table 4) represent the deprotonated and protonated molecular ion of the 290.271 $g{\cdot}mol^{-1}$ catechin molecule, respectively. Experiments on the solubility of catechin in water/ethanol mixtures demonstrated a direct proportional relationship between the solubility of catechin and both temperature and ethanol ratio (Cuevas-Valenzuela, González-Rojas, Wisniak, Apelblat, and Pérez-Correa, 2014). This allows for a broad selection of ethanol aqueous mixture solvents to target specific bioactives in willow bark. Catechin has also been studied for its anti-inflammatory properties, therefore it is one of the compounds responsible for the beneficial effect of willow bark extracts. In a clinical experiment, two groups underwent examination: control group and group suffering from prostatitis. Catechin and nanocatechin supplements were administrated to the infected individuals. By assessing the degree of inflammation in the examined groups, results showed that individuals who were given catechin or nanocatechin supplements produced higher anti-inflammatory and antibacterial responses than the control group (Yoon et al., 2011). Other studies have proven the efficacy of catechin in preventing dental inflammation such as pulpitis by reducing the activity of the lipopolysaccharide (LPS) and peptidoglycan (PG) factors (Nakanishi et al., 2010).

Other compounds identified in this study such as luteolin, kaempferol and apigenin were also evaluated for their antioxidant and anti-inflammatory properties. According to Tian et al, 2021, luteolin and apigenin showed strong antioxidant activities, while kaempferol produced the highest anti-inflammatory response amongst (Tian et al., 2021). In addition, apigenin, similar to salicortin, hindered inflammation response caused by the activity of lipopolysaccharide (LPS) in macrophages (Zhang, Wang, Gurley, and Zhou, 2014).

A clear difference between the bark and wood fractions of the tested willow varieties was noted in terms of bioactives composition. Catechin and triandrin were the only two major compounds identified in the wood fraction. In fact, the presence of triandrin in wood, compound typically found in Salix Triandra, provide the answer to the proved anti-inflammatory effect of willow branches and pulp extracts, and not merely bark extracts, increasing the potential use of the entire willow plant for medicinal purposes (Sannikova, Popova, and Kompantseva, 2018). However, the polyphenolic compositional difference between wood and bark warrants for their separation prior to transformation processes (Borrega, Pihlajaniemi, Liitiä, Wikström, and Tamminen, 2021), since the presence of bark might interfere with hydrolysis pre-treatments and therefore inhibits the production of glucan to obtain cellulose (Li et al., 2016). The presence of polyphenols such as catechin and triandrin in willow pulp might be of interest since the request for high-performing biomaterials has dramatically increased in recent years. In fact, anti-inflammatory and antimicrobial compounds are being added directly into food packaging and have improved the conservation of foods (Bouarab Chibane, Degraeve, Ferhout, Bouajila, and Oulahal, 2019). The natural presence of such compounds in the willow pulp can therefore be considered as an advantage.

The mechanism of action of willow bark extracts has been thoroughly investigated to point out the main player in its anti-inflammatory action. For instance, the DPPH levels in *Salix Purpurea* and *Salix Viminalis* extracts, which are tabulated in Table 6 demonstrate their antioxidant and anti-inflammatory effectiveness, however a compound which is majorly responsible for this action has not been singled out (Dudonne et al., 2011).

Table 5
Identification of high-value constituents in bark of Terranova, Endurance, and S. Purpurea varieties under negative and positive ionization mode (-/+ESI).

Constituents	$[M-H]^-$ (m/z), $[M-H]^+$ (m/z) and other fragments *Terranova* Bark (-)	*Terranova* Bark (+)	*Endurance Bark (-)*	*Endurance Bark (+)*	*S. Purpurea* Bark (-)	*S. Purpurea* Bark (+)
Catechin $C_{15}H_{13}O_6$ 290.26 $g.mol^{-1}$	X *289.0760 [M-H]-*	X *291.0851 [M+H] +*	X *289.0782 [M-H]-; 179.0606*	-	X *289.0760 [M-H]-*	X *291.0903 [M+H]+*
Salicin $C_{13}H_{17}O_7$ 286.28 $g.mol^{-1}$	X *285.1060 [M-H]-; 311.1121 formate adduct*	X *309.0929 [M+Na] +*	X *285.1046 [M-H]-; 311.1102 formate adduct*	X *309.1098 [M+Na] +*	X *285.1024 [M-H]-; 311.1079 formate adduct*	-
5-Methoxysalicylic acid $C_8H_8O_4$ 168.04 $g.mol^{-1}$	-	-	-	-	X *213.9676 [M-H + HCOOH]*	-
Salicortin $C_{20}H_{23}O_{10}$ 424.4 $g.mol^{-1}$	X *423.1765 [M-H]-*	-	X *423.1383 [M-H]- '469.1442 [M-H + HCOOH] −*	-	X *423.1363 [M-H]-; 469.1416 formate adduct*	-
Acacetin-5-O-xyloside $C_{21}H_{19}O_{19}$ 416.1 $g.mol^{-1}$	-	-	X *415.2059 [M-H]-*	-	-	-
Luteolin-7-glucoside $C_{21}H_{20}O_{11}$ 448.10 $g.mol^{-1}$	-	-	X *447.1593 [M-H]-*	-	-	-
Picein $C_{14}H_{19}O_7$ 298.291 $g.mol^{-1}$	-	X *299.1091 [M+H] +*	-	X *299.1241 [M+H]+*	-	-
Kaempferol-7-O-glucoside $C_{21}H_{20}O_{11}$ 448.38 $g.mol^{-1}$	X *447.1044 [M-H]-; 463.0988*			-	-	-
6-Prenylnaringenin $C_{20}H_{19}O_5$ 340.13 $g.mol^{-1}$	X *339.2096 [M-H]-*	-	-	-	-	-

Table 6
Antioxidant activity of S.Viminalis and S. Purpurea bark varieties (Dudonne et al., 2011).

Willow Variety	DPPH (gTrolox/g d.m.)[a]	ABTS (%)[b]	EC50 (mg/cm3)[c]
S. Viminalis (bark)	4.67	32.25	5.14
S. Purpurea (bark)	2.20	21.36	5.52

[a] 2,2-diphenyl-1-picrylhydrazyl
[b] 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfuric acid)
[c] half maximum effective concentration

For this reason, extensive analysis of aqueous willow bark extracts was performed by Nahrsted et al, 2007, to determine at least the classes of compounds that contribute to the overall beneficial effect. The findings showed that the medicinal effect of the crude willow bark extract is attributed to the polyphenols and flavonoids, while salicin contribution was only minor. This comes in harmony with another study, where salicin contributed only to 0.03% of the anti-oxidant activity of the poplar bark extract (Dudonne et al., 2011). Nahrsted and his team also demonstrated the efficacy of willow bark extract as a natural substitute of aspirin, reducing common side effects of acetylsalicylic acid (Nahrstedt et al., 2007). A recent experiment comes in agreement, stating that the presence of polyphenolics with strong antioxidant and anti-inflammatory properties might play a major role in the beneficial effects provided by the consumption of willow extracts (Shara and Stohs, 2015).

In a separate study, the authors of this manuscript also investigated the effect of soil nutritional values on salicin concentration in willow bark. The experiment consisted in determining the concentration of salicin in several willow varieties grown in two compositionally different soils. Analysis was performed by using high performance liquid chromatography equipped with UV detection. In fact, the anti-inflammatory effect of the total crude willow bark extracts is not exclusively dictated by the concentration of the high value constituents (salicin, polyphenols, and flavonoids) but by the composition and fertility of the soil (Warmiński, Stolarski, Gil, and Krzyżaniak, 2021).

It is worth noting that possible damaging of DNA was recently investigated by (Maistro et al., 2022) with the administration of *Salix Alba* bark extracts to albino mice on a course of a 7 days using different intakes of 500, 1000, and 2000 mg/Kg of body weight. Afterwards, a comet assay aimed at performing genotoxicity analysis on mice blood leukocytes, liver, bone marrow, heart, and testicular cells was used. Results clearly showed that *Salix Alba*'s extract did not provoke any DNA damaging for any of the examined cells.

Conclusion

Several common varieties of willow in the Northern European climate were assessed for the presence of high-value bioactive constituents. Constituents such as salicortin, catechin, apigenin, acacetin, triandrin and salicin were identified. A clear compositional difference in terms of bioactive ingredients was firstly noted between the pulp (stem wood) and the bark of the willow species. Higher incidence of valuable constituents was determined in bark. However, the presence of these constituents in the pulp might improve the quality of food bio-packaging produced from the treatment of willow pulp in terms of conservation and protection from external agents. In addition, this research focuses on ethanol-based extracts of willow bark and wood due to their higher efficiency compared to water extracts rich in bioactive salicin. Therefore, the total anti-inflammatory effect of willow bark is not exclusively attributed to the activity of salicin since the presence of polyphenols and flavonoids as the ones identified in this research possibly play a major role in the total anti-inflammatory effect of willow.

CRediT authorship contribution statement

Ayman Hijazi: Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Italo Pisano':** Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review &

editing, Visualization. **J.J. Leahy:** Methodology, Supervision. **Witold Kwapinski:** Methodology, Supervision. **Christopher R. Johnston:** Writing – review & editing. **John Prendergast:** Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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