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Original Article

GENDER SPECIFIC VARIATION OF TWO PHENOLIC GLYCOSIDES (POPULIN AND SALICIN) IN *POPULUS CILIATA* AND IDENTIFICATION OF A NEW COMPOUND (CINNAMOYL-SALICIN)

AMITA KUMARI[1]*, NAVNEET K. UPADHYAY[2], PREM K. KHOSLA[1]

[1]School of Biological and Environmental Sciences, Shoolini University, Solan, India, [2]Faculty of School of Pharmaceutical Sciences, Shoolini University, Solan, 173229, India
Email: amitabot@gmail.com

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ABSTRACT

Objective: To observe gender specific seasonal variation of two phenolic glycosides (PG's) (populin and salicin) in *Populus ciliata* male and female trees.

Methods: Plant material (bark) was collected from male and female trees throughout the year. The content of salicin and populin was measured using HPLC. Because of the lack of populin in the market, the standard compound was synthesized in the laboratory from salicin following standard procedure. Confirmation and characterization of synthesized populin were done using ^{1}H NMR and ^{13}C NMR. TLC and LC-MS of methanolic extract were performed to observe the presence of populin and salicin in the plant bark.

Results: TLC showed the presence of populin and salicin in crude plant extract at Rf value 0.84 and 0.52, respectively. The results of monthly variation showed a consistent pattern of two PG's for both the sexes. However, salicin content was observed highest compared to populin content. Whereas female trees were observed with low content of PG's compared to male trees. The maximum content of salicin and populin was observed in the flowering season. LC-MS of bark methanolic extract confirmed the presence of a significantly larger peak, which was identified as a cinnamoyl-salicin peak at 463.

Conclusion: The study confirms the highest content of PG's in the flowering season. Additionally, LC-MS study concluded a new compound cinnamoyl-salicin (M^{+}at 463) which is reported first time to the best of author's knowledge. It seems that it could be the denaturation product of 2'-O-cinnamoyl salicortin and can be further explored for biological applications.

Keywords: Phenolic glycosides, *Populus ciliata,* salicin, Populin, Cinnamoyl-salicin, TLC, NMR, HPLC

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INTRODUCTION

Secondary metabolites, particularly phenolic compounds, are bioactive compounds in the plants which include lignins, anthocyanins, salicylate-like phenolic glycosides (PG's) and tannins [1]. The role of PG's has been found to be of defense mechanism against herbivores (gypsy moth) and as chemotaxonomic marker [2, 3]. Additionally, they have also medicinal value, e. g., salicin is hydrolyzed and oxidized into salicylic acid after ingestion, having a similar action as to aspirin [4]. Therefore, PG's like salicin and populin synthesizing plants are now mostly explored by pharmaceutical companies for their medicinal values.

PG's are widely distributed among the members of family Salicaceae including genera *Populus* [5]. But the content and type of the PG's are found to be different among *Populus* species [6-8]. The variation in the amount of PG's can be accounted to environmental conditions, plant gender or clonal differences and influences the plant susceptibility to insects [9-12]. Many authors have worked on dioecious plants in relation to seasonal variation in PG's, but results were not consistent. Recently, it has been hypothesized that PG's play a significant role in gender determination and regulation of floral pathogens which coevolved with resistant to the floral pathogen. Both these events, i.e., floral pathogen and resistance to floral pathogen, triggers the emergence of a nascent sex chromosome [13].

Populus ciliata Wall ex. Royle, commonly known as Himalayan Poplar is rich in many PG's. A total of 38 phenolic compounds were identified in bud exudates of *P. ciliata* by Gas chromatography-Mass spectrometry (GC-MS) [14]. However, most of the work on seasonal variation and concentration of PG's in relation to herbivore was done on foliage in dioecious plants. The concentrations of primary and secondary foliar metabolites were reported showing temporal variations affecting the performance of associated herbivores [15-17]. No literature was found in which seasonal or monthly variations in the bark of *Populus* is reported. Therefore, the present study was the preliminary step towards the gender-specific seasonal variation in two PG's (salicin and populin) in *P. ciliata* trees.

METHODS AND MATERIALS

Chemicals and reagents

For the present study chemicals and reagents used were: methanol (HPLC grade), ethyl acetate (HPLC grade), formic acid (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade) from High Media, India; benzoyl chloride and salicin from Loba, India and populin (prepared in Shoolini University laboratory).

Instruments

The instruments used were: TLC plates (Silica gel 60 F254b TLC Aluminium sheets 5x20 cm) from Merk Millipore, India; Ultra Violet (UV) Chamber Unicorn, India, Reverse phase high pressure liquid chromatography (RP-HPLC) of Agilent technology, Fourier transform infrared spectroscopy (FTIR), India; Bruker Avance II 400 NMR Spectrometer SAIF.

Site description and tree identification

Male and female trees of *P. ciliata* in triplicates were identified and marked during the flowering season (March-April) from district Shimla (near Sankat Mochan Temple), India. The sampling site is situated at 31° 06' N / 77° 13' E, northern India. The average elevation is 1975 m above sea level.

Collection and preparation of plant material

The bark of male and female trees (Voucher specimen number SUBMS/BOT-5223/5224) were collected every month for one year, washed under tap water and dried in an oven at 30 °C till it attained consistent weight. The dried bark was then crushed and stored in

the coarse powder form in polyethylene bags. The powder was further used for methanolic extract preparation and to observe monthly variations of PG's using HPLC.

Preparation of methanolic extract

For the preparation of methanolic extract, 5 g dry plant powder was dissolved in 50 ml of methanol and extracted using soxhlet for 24 h at 50 °C. The extract was then filtered and stored at 4 °C in airtight bottles for further use.

Chemical synthesis of populin [18]

Populin was synthesized chemically in a laboratory from salicin. 500 mg of salicin was dissolved in 10 ml of distilled water. 0.625 ml of benzoyl chloride was added to the solution drop by drop in the course of about half an hour, during that time the mixture was stirred vigorously and kept alkaline with the addition of 1N aqueous potassium hydroxide.

A white, granular solid product (benzylated derivatives) was formed which was filtered and washed well with water. The populin was separated from benzylated derivatives by extraction with boiling water. The boiled water was then filtered and kept in the refrigerator for one day. Needle-like crystals appeared in the cold water. Water was decanted, and crystals were washed with alcohol for purification purpose and produced good-sized prisms which were solvent free. The final yield was found to be only 220-350 mg (fig. 1).

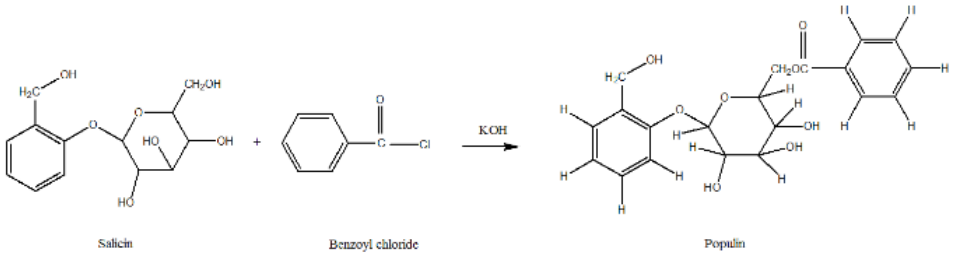

Fig.1: Chemical reaction of populin synthesis

Qualitative determination of salicin and populin

For qualitative determination of salicin and populin in methanolic extract of bark, TLC was used with two solvent systems, ethyl acetate: methanol (70:30) and methanol: water (70:30). Commercial salicin and synthesized populin were used as a standard. For applying test samples on TLC plate, glass capillaries were used. The spots were applied with the help of a transparent template, keeping a minimum distance of 1 cm between the two adjacent spots. The spots of the samples were marked on the top of the plate to know their identity. Resolution factor (Rf) was calculated as given below-

$$Rf = \frac{\text{Distance travelled by the solute from the origin}}{\text{Distance travelled by the solvent front from the origin}}$$

Quantitative determination of concentration of salicin and populin [1]

Determination of phenolic PG's was performed using instruments such as HPLC system of Agilent technology composed of bin pumps combined with Agilent technologies ALS along with photodiode array detector with a column from Agilent eclipse XBD® C_{18} bonded with 5 µm (4.6 x 150 mm) coupled with EZ-Chrome software recording. System suitability parameter was calculated before starting validation parameters. It was determined by taking % RSD (relative standard deviation) of the five standard injections using the same concentration of two PG's (salicin and populin) by HPLC method which indicated the performance of the HPLC instrument under the chromatographic conditions. As a part of method validation minimum, five injections of the standard preparation were performed for inter-day precision. The relative standard deviation was not more than 2 %. Limits of detection and quantification were calculated by a method based on standard deviation (σ) and slope (S) of calibration plot using formula Limit of detection (LOD) = 3.3 σ/S and Limit of Quantification (LOQ) = 10 σ/S. The mobile phase combination of acetonitrile and water of HPLC grade (with 0.1 % formic acid) in the ratio of 40:60 v/v with RP-HPLC at a flow rate of 0.5 ml/min was found to be most suitable. Best resolution and sensitivity of the method was obtained for PG's (salicin and populin) was 254 nm. A calibrated curve of salicin and populin was prepared separately using different concentration (1, 2, 3, 4 and 5 µg/ml) of pure salicin and populin. Typical chromatogram with optimized condition gave sharp and symmetric peak with specific retention time of 11.06±0.91 (salicin) and 21.03±0.07 (populin) minutes for females and 12.00±0.031 (salicin) and 20.95975±0.10 (populin) minutes for males (fig. 2 a,b). The content of salicin and populin was calculated by linear regression.

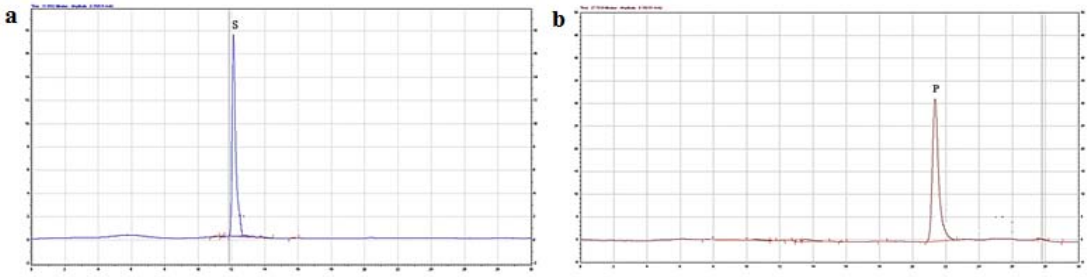

Fig. 2: Chromatogram of standard salicin (a) and populin (b) showing peak with Rt-12 and 21 at 254 nm, respectively

RESULTS

Characterization of synthesized populin

The synthesized sample showed needle-like white colored crystals (m. p.-179°C lit. 180°C) with light sweetish taste. The confirmation of the compound was also done with conc. H_2SO_4 which produced light pinkish color. The physical characterization proceeded with FTIR (fig. 3), Proton nuclear resonance spectroscopy (^{1}HNMR) (fig. 4) and Carbon-13 nuclear magnetic resonance (^{13}CNMR) (fig. 5).

FTIR results

In FTIR of populin, the values 3376 cm^{-1} and 3294 cm^{-1}correspond to stretching frequencies of O-H groups. The spectrum also showed stretching frequencies at 2933 cm^{-1} and 2884 cm^{-1} due to

asymmetrical and symmetrical stretching of C-H and 1050-1250 cm^{-1} due to C-O stretching, respectively. The peaks at 1450, 1500-1650 cm^{-1} are attributed to the presence of aromatic ring C-C stretching. Additionally, the FTIR spectrum of populin has an intense, sharp peak at 1719 cm^{-1} which is attributed to the presence of the a-COOR group in contrast to the salicin FTIR (figs. 3 a,b).

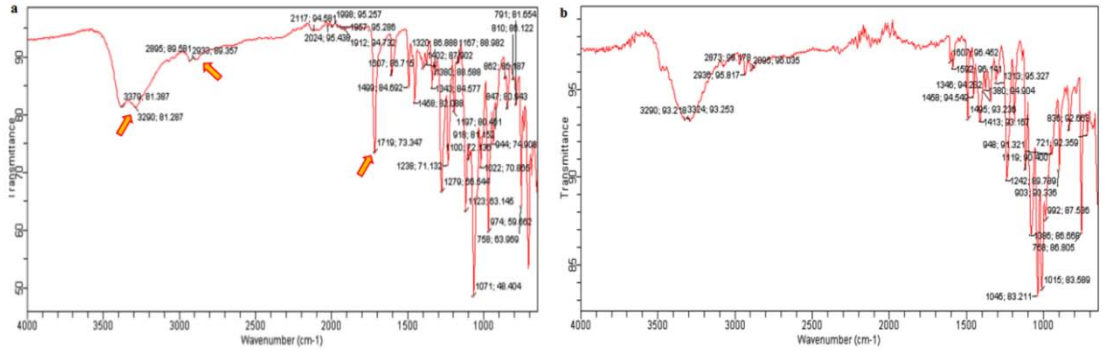

Fig. 3: FTIR of populin (a) and salicin (b)

^{1}H NMR results

^{1}H NMR results are shown in fig. 4. Populin was synthesized from salicin. Structurally salicin is composed of two main parts; one is salicyl alcohol (aromatic part) and other is sugar part. Therefore, signals obtained by the ^{1}H NMR spectroscopy should lie in two regions first one was the aromatic proton that is much down-field i.e. 6-8 ppm. Another part gave values typical of oxygenated and other aliphatic protons and was in the up-field. All values are presented in δ; DMSO. The major difference between salicin and populin ^{1}HNMR is clearly understandable. Populin showed extra signals for benzoate five protons to confirm the successful reaction as given in table 2.

Besides, the significant observation, C-7' methylene protons of sugar moiety showed characteristic peak shift from 3.35 and 3.66 to 4.35 and 4.67, respectively as expected due to the formation of the ester linkage. The rest of the signal was more or less same as that of the salicin.

Table 1: Different signals of ^{1}H NMR of Salicin

S. No.	Proton	Chemical Shift (ppm)	Type	J (Hz)	
1.	2	7.13	d	7.32	
2.	3	7.23	T	8.20	
3.	4	6.99	T	7.32	
4.	5	7.30	d	7.45	
5.	7	4.43, 4.74	d	Geminal coupling	
6.	8	4.77	d		
7.	9	3.40	T		Sugar proton
8.	10	3.30	T		
9.	11	3.46	T		
10.	12	3.80	d/T	11.24	
11.	13	3.50	d		

Table 2: Extra signals of ^{1}H NMR of Populin

S. No.	Proton	Chemical Shift (ppm)	Type	J (Hz)
1.	2'	8.01	d	7.36
2.	3'	7.51	T	7.70
3.	4'	7.64	T	7.30
4.	5'	7.51	T	7.70
5.	6'	8.01	d	7.36

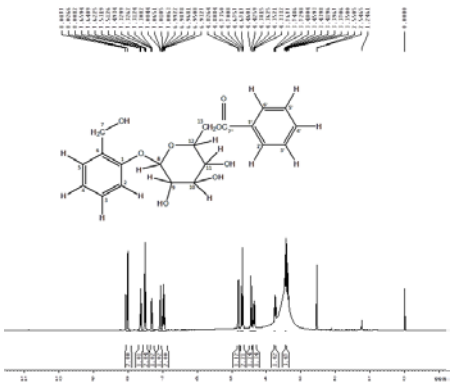

Fig. 4:^{1}H NMR of populin

^{13}C NMR results

^{13}C NMR spectroscopy supports populin stretching as depicted by ^{13}C NMR results with characteristic peaks of salicylic alcohol units between 58 to 155 ppm (C-1, 154.88; C-2, 132.91; C-3, 129.65; C-4, 121.87; C-5, 128.26; C-6, 114.90; C-7, 58.89). The intense peak at 58.89 ppm was the characteristic of primary alcohol present in salicylic alcohol. The peaks for sugar unit of populin appeared between 58 to 101 ppm (C-1′, 101.54; C-2′, 73.21; C-3′, 76.29; C-4′, 70.11; C-5′, 76.29; C-6′, 66.09), where anomeric carbon (C-1′) in sugar unit was evaluated at 101.54 ppm. Additionally, populin also showed extra signals in the aromatic region for benzoate five protons at substituent C-6" of sugar unit between 114 to 165 ppm. The peak at 165.37 ppm was associated for ester linkage between benzoic acid and sugar unit of populin (fig. 5). The above characterization results showed that the synthesized compounds was populin having molecular formula $C_{22}H_{20}O_8$.

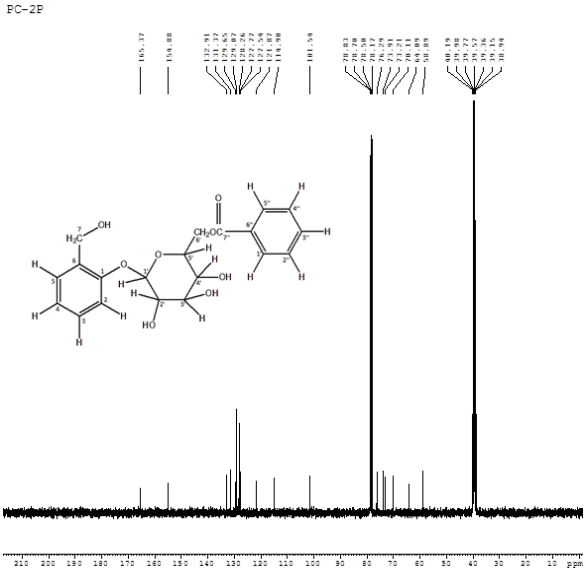

Fig. 5: ^{13}C NMR of populin

TLC of methanolic extract of bark

Different spots were generated on silica gel plate with different Rf values, and the identification of compounds was done by comparing the extract with standards (salicin and populin) (fig. 6). The Rf value with different solvent systems for salicin and populin are shown in table 3. These solvent systems can be used to separate the salicin and populin from the plant parts at the industrial level.

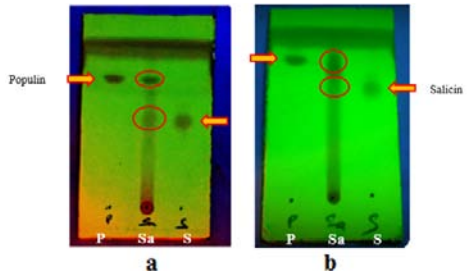

Fig. 6: TLC plate showing bands of PG's in bark of *P. ciliata* methanolic extract (Sa) with standard populin (P), salicin (S) in solvent system ethyl acetate: methanol (70:30) (a); methanol: water (70:30) (b)

Table 3: Rf values of salicin and populin in two different solvent systems

S. No.	Solvent system	Ratio	Rf value	
			Salicin	Populin
1.	Methanol: water	70:30	0.52	0.84
2.	Ethylacetate: methanol	70:30	0.48-0.68	0.74-0.83

Gender specific variation of salicin and populin in the bark extract of *P. ciliata*

In all the months (January to December), salicin and populin had similar two to four dominant sharp and zero to two small peaks, out of which two (one dominant and one small) peaks were known. The variation was observed in the concentration of salicin and populin content only. Keeping this fact in view only one month (March) chromatogram is explained herewith.

The chromatogram of male trees was characterized by the presence of three sharp and seven small peaks, of these only two were known. The peak of salicin is marked by 'S' at Rt = 11.84, and populin is represented by 'P' at Rt = 21.39 (fig. 7b). The observed salicin content was 2.46 mgg^{-1}DW, whereas populin content was 0.016 mgg^{-1} DW. On the other hand, the chromatogram of bark of female trees was characterized by the presence of approximately two-three unknown and one known sharp dominant peaks and another five to six short peaks, of which only one was known (fig. 7a). Peaks of salicin and populin are indicated by 'S' at Rt =11.99 and 'P' at Rt = 20.93, respectively. The content of salicin was found to be 1.816 mgg^{-1}DW and of populin 0.012 mgg^{-1}DW.

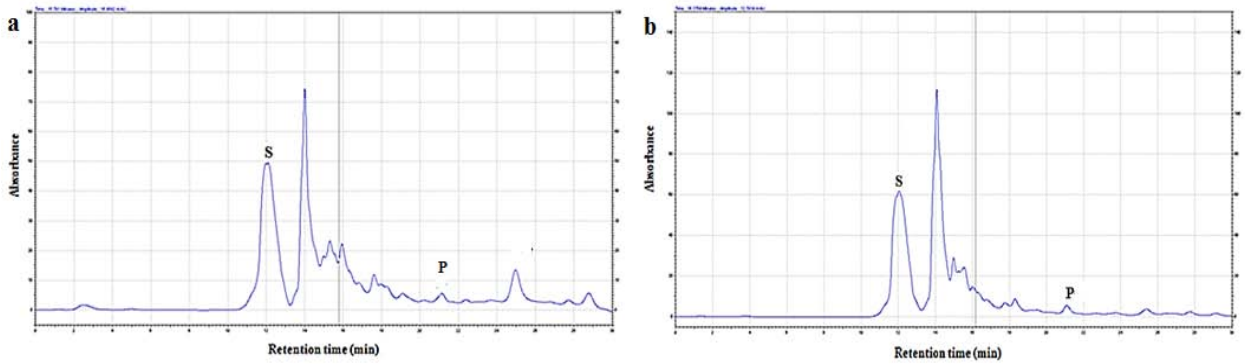

Fig. 7: HPLC chromatogram of male (a) and female (b) trees of *P. ciliata* in the month of March (S-salicin peak and P-populin peak)

Monthly status of salicin and populin content

Salicin content

The monthly status between male and female trees regarding salicin content is shown in fig. 8. Starting from September to April it increased and from April to August it declined. This trend was observed equally in both male and female trees. The Maximum content of salicin was observed in April in both female (0.2806 mgg^{-1}DW) and male trees (0.43664 mgg^{-1}DW); whereas it was minimum in September in female (0.0170 mgg^{-1}DW) and in August in male trees (0.5312 mgg^{-1}DW). However, male trees bark had more salicin content in all the months compared to female trees bark.

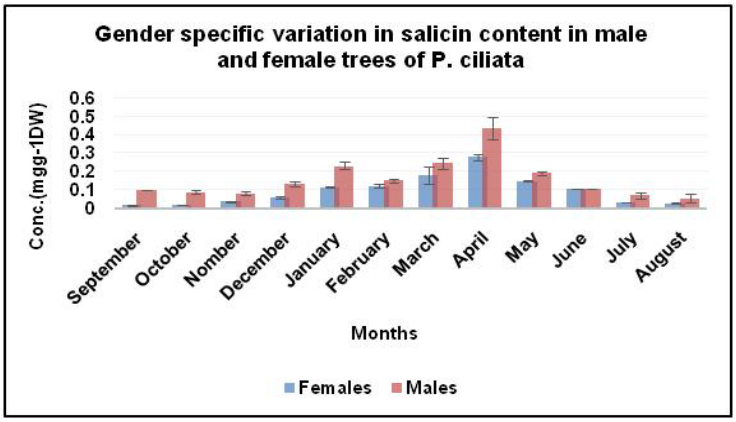

Fig. 8: Comparison in monthly variation in concentration of salicin content (mgg^{-1}DW) in three male and three female trees of *P. ciliata*

Populin content

The monthly analysis of populin content is shown in fig. 9. Male and female trees revealed differences in populin content, being higher in male trees. It was maximum from September to April (0.0095 mgg^{-1}DW) in male trees. The lowest content of populin was in June, while in remaining months it was absent. On the other hand, in female trees from January to May populin was present while in another month it was absent. In July populin production was insignificant (0.0001 mgg^{-1}DW). The maximum concentration of populin in female trees was observed in March (0.0137 mgg^{-1}DW). This difference was found to be prominent in both male and female the trees. However, in male trees, populin content was also found between September to December and also in June. In females, populin content was found to be totally absent. Monthly variation of populin content in both trees also revealed similar pattern from January to April while in other months pattern was different.

The comparison of salicin and populin content revealed that the concentration of salicin and populin content was higher in males than in females. Salicin production, in general, was higher than the populin in both the sexes of trees.

Liquid chromatography and mass spectrometry study

Methanolic extract of bark was used for LC-MS study to observe the presence of salicin and populin. A significant larger peak was observed using a solvent system acetonitrile with 0.10 % formic acid and distilled water as a cinnamoyl-salicin peak at 463 (M-H+HCOOH+H i.e. 416+46+1) (fig. 10).

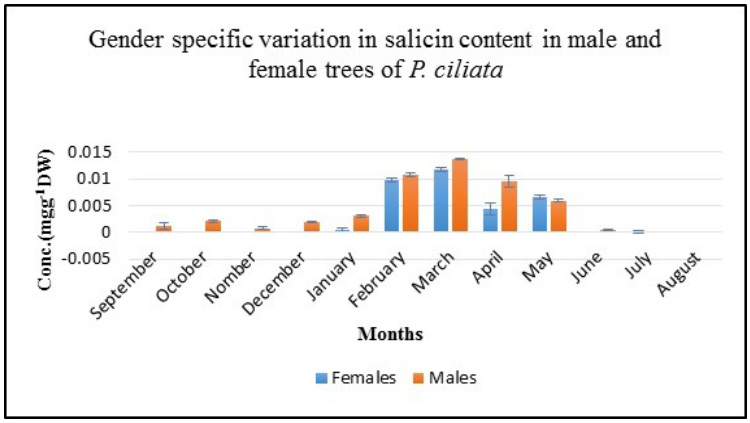

Fig. 9: Comparison in monthly variation in concentration of populin content (mgg^{-1}DW) in three male and three female trees of *P. ciliata*

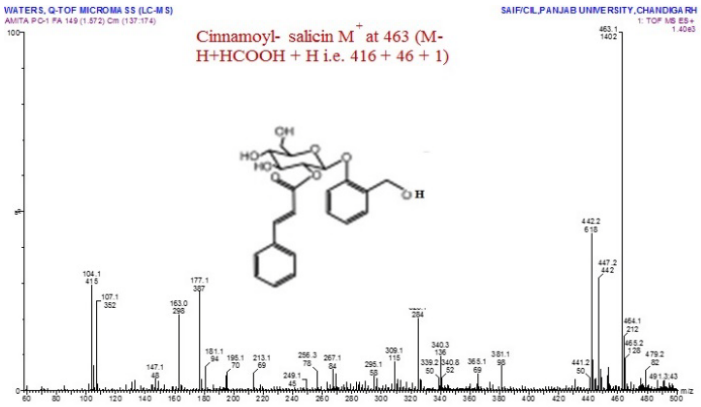

Fig. 10: LC-MS of bark extract

DISCUSSION

HPLC is a powerful technique used for the identification, separation, and quantification of individual components of a mixture. The technique has also been successfully used for the identification and quantification of various bioactive compounds in plants and endophytic fungi, e.g., steroids from *Portulaca quadrifida*, swertiamarin from *Enicostemma littorale*, mangiferin from *Mangifera indica*, PG's from *Salix sericea* and terpenoid from *Pestalotiopsis* sp., respectively [11, 19-22]. In the present study, HPLC analysis showed increased salicin content from September to April and then from April to August it declined. On the other hand, populin was observed to be maximum from September to April in male trees and January to May in female trees. The results showed the maximum content of these PG's during the spring season and minimum in autumn season. A similar pattern of PG's variation was observed from the bark of *Salix* [11, 23]. Results also support the accumulation of PG'S in autumn [13, 24]. Whereas, some authors reported a decrease of PG's from winter to summer [25].

The present study also deals with the variation between the concentration of two PG's between male and female trees. Such type of variation was previously reported among plants in the total level of PG's [2,7]. The comparison of salicin and populin content revealed that the concentration of salicin and populin content is higher in males than in females, which could be the reason of lower susceptibility of male towards floral pathogens. In general, the production of salicin is found to be higher than the populin production in both the sexes. Such type of finding was also observed in *Rumex acetosella* males with a higher concentration of PG's than females [26]. The reason behind the low concentration of PG's in females is that females expend more energy in fruits production and maturation, which reduces the concentration of PG's [27]. On the other hand, contradictory results were reported in some willow species where male plants had lower concentrations of PG's than females [10, 9]. The same can be accounted to the more susceptibility of males to herbivory and pathogens because they invest a greater percentage of their resources to growth [17, 27].

Monthly variation of populin content in both sexes showed the similar pattern from January to April while in other months pattern was different. On the other hand, variation in salicin content was observed almost similar in both male and female trees, but it was not consistent in both sexes. Furthermore, the level of both PG's was observed highest in a flowering season (from February to April). However, populin did not show a regular pattern of variation, which was also confirmed by previously reported studies [11]. The salicin content observed in the study revealed that salicin is the product of biosynthesis reaction, therefore, present as a major compound in the family members of Salicaceae (*Populus* and *Salix*). On the other hand, populin was reported from only a few species of *Populus*, i.e., *P. grandidentata* and *P. tremuloides,* etc. [18, 28]. The limited range of distribution of populin in *Populus* species and its irregular variation opposes the previously reported finding that populin exists in aspens as a product, not an intermediate compound. Whereas, our results support the presence of poplin as an intermediate compound. The stability and the turnover rate of these PG's have been reported to be affected by some factors like mechanical damage [29, 30], exposure to foliar enzymes (like esterase and β-glucosidase) and complexity of the molecule [31] which can account for irregular results. Moreover, the seasonal change in PG's in plants can be supported due to available photosynthate which peaks over the summer due to the maximum leaf area and reduced tissue production rate [11].

A new compound (cinnamoyl-salicin) was identified during the study from the bark, but its role in the plant has not been yet confirmed. The position of location of salicinnate could not be ascertained due to the absence of NMR of the pure compound. The presence of this molecule appears to be new to literature as no such molecule has been reported so far. It seemed that it may be the denaturation product of 2'-O-cinnamoyl salicortin as reported in *P. tremula* [1].

CONCLUSION

Present study proves the accumulation of PG's during flowering season in both sexes, which would create a possibility of their role in flowering and therefore create a new area of research. A new compound was identified (cinnamoyl-salicin) during the study, which is yet not reported in the literature. Further investigation requires to separate or purify that compound from the plant extract and should be screened to its medicinal and other industrial values.

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CONFLICTS OF INTERESTS

Declared none

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