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Phytopharmacognostic profiling of *Prunus cerasoides* Buch.-Ham. ex D. Don, heartwood

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Use of *Prunus cerasoids* Buch.-Ham. ex D. Don is mentioned in traditional texts as *padmak* with some of its medicinal values. Seeing the classical significance of the plant, the study was undertaken to develop a pharmacognostic and phytochemical blueprint of *Prunus cerasoids* heartwood. The primary goal of this study was to detect the bioactive flavonoids, like biochanin A, genistein and sakuranetin, in heartwood. Authenticated plant materials were subjected to pharmacognostical, physicochemical and HPTLC fingerprinting. Qualitative analysis in detecting phytocompounds in the extracts of varied solvent polarity was performed using LC-MS/MS orbitrap. The extraction efficiency was highest in polar organic solvent methanol, and LC-MS/MS ascertained the same. The significant outcome of this study was the extractability of the solvents in bringing down the active phytochemicals differently, and methanol was found to be the best-suited solvent. Maximum numbers and the major phytocompounds available were apigetrin, astilbin, betaine, biochanin a, caprolactum, catechin, choline, coumarin, eriodictyol, ethylmalonic acid, formononetin, genistein, glycitein, hematoxylin, naringenin, phloretin, pipecolic acid, prunin, quercetin, rutin, sakuranetin, taxifolin, and trifolin. The data set generated here had multi-faceted contributions, especially in phytopharmaceuticals. This multidimensional profile of the plant heartwood may serve as documentary evidence in preparing a genuine monograph.

Keywords: Flavonoids, HPTLC, LC-MS/MS, Microscopy, *Prunus cerasoides*

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Introduction

Prunus cerasoids Buch-Hem. ex D Don, under the family Rosaceae, is naturally a habitant of the temperate Himalayan region extending from Kashmir, Himachal Pradesh, Uttarakhand to Sikkim, Bhutan, with an altitude of 4000-5000 feet high, known as bird cherry, wild Himalayan cherry or sour cherry in English[1-6].

The plant is mentioned in the traditional text as *Padmak*. The different parts of *Prunus cerasoides*, like heartwood, stem bark, seed, seed oils, etc., have been used for therapeutic purposes for immemorial time[3,4]. As per Ayurvedic pharmacology, the plant is *Kasaya* (astringent), *Tikta* (bitter), *Sheetavirya* (cold potency), *laghu* (mild) and *Snigdha guna* (soft). It transformed as *Katu* (unpleasant) after *vipaka* (metabolism) with *vedanasthapak* (pain reliever) as a special character[7-10]. It is specifically used in *Raktapitta* (bleeding disorders), skin diseases like boils, wounds, chronic lesions, nausea and vomiting, hiccups, and excessive thirst either in single or in combined with other ingredients[11-12]. Many preparations like *Asava Arista*, *Arka*, *Kwathchurna*, *Ghrita*, *Taila*, *Vati*, *Gutika* etc., with *padmak*. Among several formulations, the uses of *Chandanasava*, *Sarivadyasava*, *Maha Kalyanaka Ghrita*, *Sudarshana, Chuma*, *Kumkumadi Taila* and *Mahaukstha Ghrita* are most common in present day[2].

Several workers carried out pharmacological and phytochemical studies in the early nineties[13-17]. A phytochemical evaluation of the plant stem was carried out, and new flavonoids were reported[18-22]. The flower and seeds of the plant were investigated for identification and isolation of its phytoconstituents[23-26]. The plant root has been studied for its phytochemical characterisation and antioxidant activities[27]. These studies revealed that the plant root, stem and sometimes seeds and flowers are rich in flavonoids like biochanin A, prunin, naringenin, genistein, sakuranetin, etc. Among all these, it has been noted that biochanin A is a multifunctional bioactive flavonoid classified as phytoestrogen, possessing antiinflammatory, anticancer, neuroprotective, antioxidant, antimicrobial,

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and hepatoprotective properties; it combats cancer development by inducing apoptosis[28]. Genistein is another important isoflavone, bearing several therapeutic activities like protection against osteoporosis, reduction in the risk of cardiovascular disease, alleviation of postmenopausal symptoms and anticancer properties[29-31]. Sakuranetin, a flavanone, is claimed to exert antiviral activity towards human rhinovirus three and influenza B virus and was reported to have antioxidant, antimicrobial, antiinflammatory, antiparasitic, antimutagenic, and antiallergic properties[32-33].

It was noted that the stem, seeds, roots, and flower parts have been explored for this plant, while the heartwood portion has not been studied, at least for pharmacognostic and phytochemical profiling. Hence, the present study undertook the pharmacognostic evaluation and phytochemical investigation of the heartwood of *P. cerasoids*. The macroscopic and microscopic studies were included for the cytomorphological characterisation. The HPTLC profiling was considered for the qualitative presence of the flavonoids. The extracts of the different solvents of the heartwood were screened for the qualitative identifications of phytochemical presence by LC-MS/MS. The output of these data contributes significantly to developing a monograph and designing a phytopharmaceutical product.

Materials and Methods

Materials and reagents

All solvents of GR grade and TLC plates used during the experiments were purchased from E. Merck Pvt. Ltd. (Mumbai, India). Standards Sakuranetin (>98% by HPLC) and Genistein (>98% by HPLC) were purchased from Sigma-Aldrich, and Biochanin A (>95% by HPLC) was procured from Fluka.

Plant materials collection and authentication

Fresh matured portions of *P. cerasoides* stem/heartwood were collected from the natural habitat of Khatyadi, District Almora, Uttarakhand (29° 53 3.2" N, 79° 18' E), in August 2022, with field book No. 42523 and accession number 29735 in the herbarium of RARI, Ranikhet, which is accredited by NY Botanical Garden Museum acronym is "RKT". Pieces of the stem (2-5 cm) were taken for study, out of which stem bark portions from the peripheral shoot region were carefully separated, and central reddish brown, closely grained, moderately hard and strong heartwood entangled with whitish sapwood was considered for the study.

Plant sample processing

The plant materials, *i.e.* heartwood fragments, were washed with aqueous 70% (v/v) ethanol and dried at an ambient temperature (24-27°C). A small portion of the air-dried plant sample was used for macroscopic, organoleptic and anatomical (transverse section) studies. In contrast, the rest of the plant materials were pulverised with a grinder (National SM 2000). The whole and powdered plant samples were stored at room temperature in airtight, light-resistant containers as per Ayurvedic Pharmacopoeia of India guidelines[34]. The finely (sieved in 60 mesh) powdered sample was used for powder microscopy. In contrast, the coarsely powdered samples were used for other experiments like physicochemical examinations, qualitative identifications of secondary metabolites and fingerprinting.

Macroscopy of plant material

The organoleptic parameters, *viz*. texture, shape, size, colour, odour, taste, etc. of the heartwood of *P. cerasoides* were noted by naked eye observation with a simple microscope (Olympus OIC DM).

Cytomorphology of plant material

The stem portion was taken for study; It was soaked in lukewarm water overnight to soften the tissues. Then, the outer layer of stem bark was carefully separated, and the inner woody portion was transversely sectioned with a new, clean, sharp-edged blade. The finely sectioned heartwood portions of the stem were mounted on slides in 50% glycerine and observed under a binocular 35 compound microscope (Olympus CX43) at 10X and 40X magnifications[35].

For powder microscopy, finely powdered samples (2 g.) were separately treated with different solutions, *i.e.* aqueous saturated chloral hydrate (for maceration), 50% glycerine, phloroglucinol in conc. HCL (for staining lignified tissues) was mounted on slides with glycerine and observed under the binocular compound microscope (Olympus CX43) with 4X, 10X, and 40X magnifications. Photomicrographs of different cellular structures were taken using a Magnam DC 14 camera attached to the microscope[36].

Physico-chemical evaluation

The physico-chemical parameters like ash values, loss on drying, extractive values in different solvents

(like hexane, chloroform, ethyl acetate, acetone, methanol, ethanol, and water) and pH value in 10% aqueous suspension of the plant material were determined as per World Health Organisation guidelines[37].

Preparation of plant extract for HPTLC fingerprinting

Extract obtained by methanol was used for the HPTLC fingerprinting because methanol gave highest extractive values among all other solvents. This coarsely powdered plant material (1 g) was extracted with 50 mL methanol using a Soxhlet apparatus. The extract was filtered and labelled as PC-M and kept for further investigations.

Preparation of standard solutions

An aliquot of 5 mg of each standard, namely, biochanin A, genistein, and sakuranetin, was dissolved individually in precisely measured 50 mL methanol.

Successive extraction of heartwood for qualitative study in LC-MS/MS

The coarsely powdered heartwood was subjected to successive extraction, starting with a non-polar solvent like hexane and ending with water as the highest polar solvent. For this, the plant material was first subjected to hot extraction with hexane (polarity index 0.1). Then, the extract was filtered off, and marc was dried and extracted with chloroform (polarity index 2.7). These steps were repeated successively with ethyl acetate (polarity index 4.4), methanol (polarity index 5.1) and lastly with water (polarity index 10.0). Each extract was filtered with a syringe filter of 0.2µm and stored in a screw cap glass bottle for qualitative study by LC-MS/MS.

HPTLC fingerprinting

The methanolic extract of the plant (PC-M) and standard solutions, each of 2 µL were applied in the form of an 8 mm band, at 15 mm from the bottom edge of a 10 x 10 cm reactivated glass supported precoated silica gel $60F_{254}$ HPTLC plate, with the help of ATS-4 applicator attached to a CAMAG HPTLC system. The mobile phase plays a crucial role during the HPTLC analysis for the exact measurement of analytes. A solvent system that would give a resolute peak with appropriate and significantly separated R_f values was highly desired. Given this, several mobile phases were tried, and gradient elution was found to have a better separation from the desired resolution than isocratic development. Hence, a two-step gradient elution was preferred with, Hexane: Chloroform: Acetone: Ethylacetate: Methanol: Acetic acid (1: 2: 1: 8: 2: 0.5, v/v) for first development up to 50 mm and with Hexane: Chloroform: Acetone: Ethylacetate: Methanol: Acetic acid (6: 1: 1: 2: 1: 0.5, v/v) for second development up to 90 mm. The plate was developed in a pre-saturated twin trough chamber. The developed plate was dried for 10 min at an ambient temperature. Images of the developed plate were captured under 254 nm. Densitometric scanning[38] of the developed plate at 254 nm was performed to identify all the standards in the sample extract.

Qualitative identification of Phytocompounds by LC-MS/MS (Orbitrap)

Previously prepared, all five extracts of heartwood obtained by successive extraction were subjected to Liquid Chromatography Tandem Mass Spectrometry LC-MS/MS orbitrap. We considered all the extracts obtained by using solvents of variable polarity to reveal the phytocompounds, mainly secondary metabolites present in a particular solvent, and to know the extractability of the solvents in bringing down the secondary metabolites, which may give a clue in the development of phytopharmaceuticals.

Instrumentation and chromatographic condition

Q-Exactive Plus Biopharma, Thermo Scientific make equipment was used for analysis and data acquisition and processing Xcalibur-Thermo Scientific, Version 42.28.14 and for data processing Compound Discoverer 3.2 SP1 were used. In the whole experiment Syncronis-C18 100 x 2.1 mm, 1.7 microns (make Thermo Scientific) column was used.

This analysis aimed to identify the secondary metabolites present in the methanolic extract of heartwood using LC-MS/MS. A Thermo Fischer Scientific make Syncronis-C18 100 x 2.1 mm, 1.7 microns was found to be suitable for analysis after trying different columns like BEHC18, CSH Fluro-phenyl, and BEH amide. Acetonitrile and methanol were tried, and acetonitrile was chosen as it gave good separation with sensitivity. Different pHs were tried: acidic, neutral, and basic; acidic pH gave good separation and sensitivity, so acidic pH was chosen, as all peaks were well resolved. Different column temperatures ranging from 30 to 50°C were tried while keeping the column temperature constant at 40°C, which was found most suitable for generating a resolute chromatogram. An isocratic program was

adopted with a mobile phase consisting of (A) 0.1% formic acid in Milli-Q water and (D) acetonitrile (40%) with a flow rate of 0.3 mL/min, keeping the runtime 35 min.

Instrumentation and Mass Spectrometry condition

QSTAR Elite LC-MS/MS equipped with ESI ion source, system was used to perform mass spectrometric analysis. The source parameters were optimised as follows: ESI voltage, 5500 V (Positive). 4500 V (Negative), nebuliser gas, 60; auxiliary gas, 50; curtain gas, 35; Turbo gas temperature, 450 C, declustering potential, 60 V (Positive), 60 V (Negative); focusing potential, 350 V (Positive), 350 V (Negative); declustering potential 2, 10 V (Positive), 10 V (Negative). Nitrogen was used in all cases. The samples were analysed using the IDA (Information Dependent Acquisition) method, which can automatically select candidate ions for MS/MS study. The orbitrap mass range was set from m/z 50 to 1000, and the mass range for product ion scan was m/z 100-900. The collision energy (CE) was set from 20 to 70 eV to optimise signals to obtain maximal structure information from the ions of interest. Accurate mass measurements of each peak from the total ion chromatograms (TICs) were obtained by the dynamic auto-calibration method, allowing for real-time internal calibration during data acquisition.

Results and Discussion

Macroscopic characters

Stem bark dark-brown smooth exfoliating in horizontal circular strips covering copper-coloured inner surface; Heartwood yellowish-brown to orange, to which some whitish portion of sapwood still attached, presence of annual rings distinctly marked by an irregular and discontinuous belt of numerous pores (Fig. 1). The texture and shape are closely grained, heavy, dense, moderately hard, very strong and reddish brown. The organoleptic study revealed that the heartwood portion is almost odourless and tasteless.

Cytomorphological characters

The transverse section (T.S.) of mature heartwood shows it consists of vessels, fibres, tracheids and xylem parenchyma traversed by xylem rays; lignified vessels, moderately thin-walled, reticulate thickening, fairly large with bordered pits having an oval-shaped, lateral perforation at each end: fibres occur mostly in groups. Usually found associated with other xylem elements, moderately thick-walled. Lumen narrow pointed at both ends; tracheids usually thick-walled, lignified elongated cells; xylem parenchyma composed of thick-walled, found associated with vessels and fibres, oval to elongated, polygonal cells, xylem rays uni- to multiseriate, uni-and biseriate, more common multseriate generally 3-5 cells wide, 40-50 cells high; (Fig. 2). Portions of heartwood, when treated with ferric chloride solution turn yellowish, pigments blue or black, indicating presence of tannin.

The heartwood powder is Reddish-yellow (Fig. 3). It shows fragments of abundant groups or single-pointed fibres, moderately thick-walled, fairly large vessels with reticulate thickening and bordered pits, thick-walled, lignified tracheid cells, pieces of ray cells and xylem parenchyma cells and an abundant amount of rosette crystals (Fig. 4). The similar cytomorphological observations are noted by some previous researchers for the stem and heartwood of the plant[39].

Physico-chemical evaluation

Evaluation of the physio-chemical parameters of the plant samples is shown in Table 1. The total ash value was 3.15%, with water-soluble and acid-insoluble ash contents at 0.78% and 1.14%, respectively, while its weight loss on drying was 0.94%. The very low moisture of *P. cerasoides* heartwood suggested that it could be stored at an ambient temperature without much spoilage. The extractive values of different solvents for the plant

Fig. 1 — Photograph of the heartwood of *P. cerasoids*.

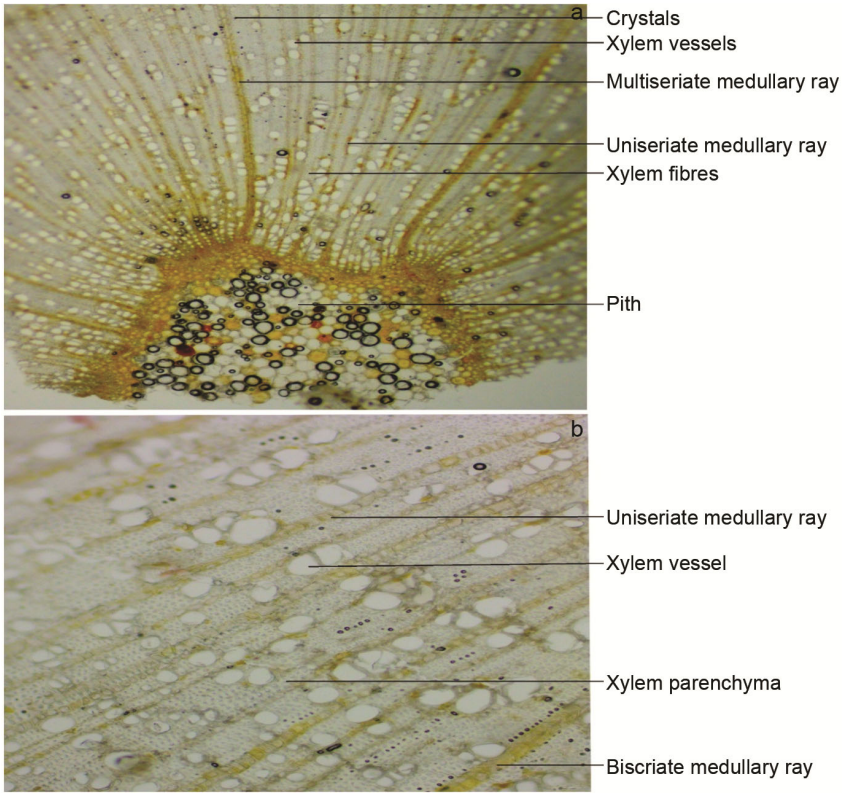

Fig. 2 — Cellular anatomy (T. S.) of heartwood of *Prunus cerasoides*. a) 4X; and b) 10X magnification.

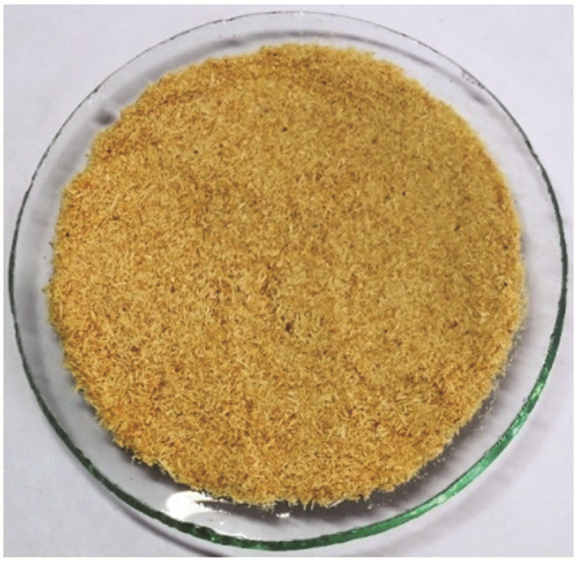

Fig. 3 — Photograph of a finely powdered sample of *P. cerasoids* heartwood.

samples revealed maximum and least extraction by methanol and hexane, respectively. The physicochemical results of the present investigations are found to be very close to the previously reported data[40]. Except for chloroform, the extraction yields of the respective solvents were not significantly different under cold and hot conditions. However, the extraction yield in chloroform was higher under hot maceration. Based on the best phytoconstituents yield in methanol extract, the same was used for the subsequent fingerprinting analyses.

HPTLC fingerprinting and identification of Flavonoids (biochanin A, genistein, sukuarnetin)

The HPTLC conditions for the best separation of the phytoconstituents were optimised for gradient development, which gave well-separated peaks with the best resolution for the phytocompounds under investigation. Methanolic extract showed bands/spots at R_f 0.11, 0.33, 0.48, 0.60, 0.72 and 0.79. The developed plate visualised under UV at 254 nm (Fig. 5) revealed the presence of Biochanin-A (R_f 0.79), Genistein (R_f 0.60) and Sakuranetin (R_f 0.72) in the plant extract. The presence of these three flavonoids was confirmed by densitometric scanning (Fig. 6). Comparison between the chromatogram of extract and the standard phytocompounds confirmed the presence of biochanin-A, genistein and sakuranetin in methanolic extract of heartwood (Fig. 7 and 8).

Qualitative identification of secondary metabolites

No one method can efficiently detect all the compounds present in herbs. Some compounds may

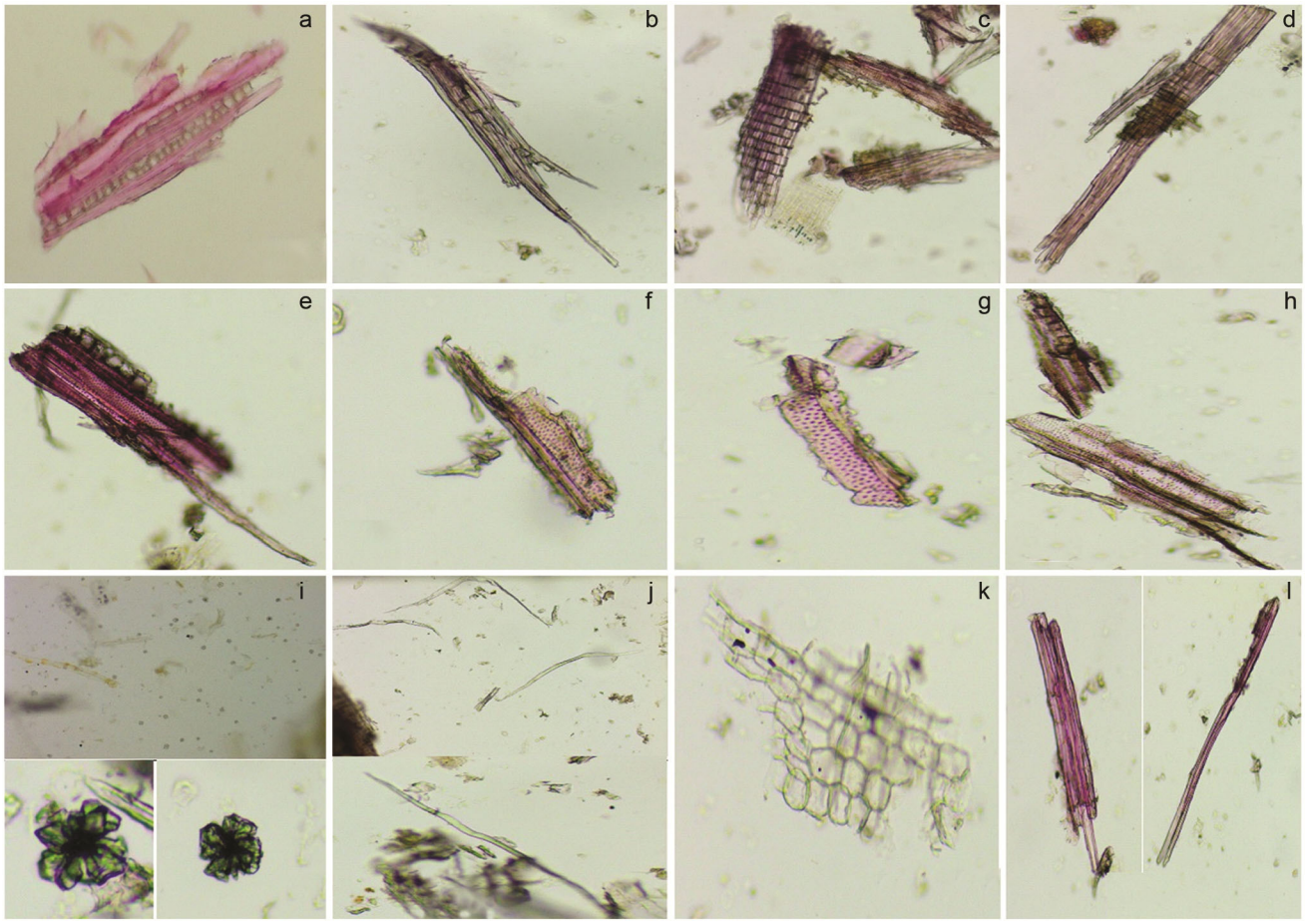

Fig. 4 — Photographs representing cytomorphological characters of *P. cerasoids* heartwood. a) Xylem parenchyma associated with tracheids; b-d) Multiseriate rays traversing on vessels; e-h) Vessels with reticulate thickening and bordered pits; i) Rosette crystal of Ca-oxalate; j) Fibres; k) Xylem parenchyma; and l) Thick-walled, lignified tracheids.

Table 1—Physico-chemical evaluation of *P. cerasoids* heartwood

Physico-chemical parameters	Weight percentage	
Loss on drying at 105°C	0.94±0.13	
Total ash	3.15±0.21	
Acid insoluble ash	1.14±0.31	
Water soluble ash	0.78±0.19	
Sulphated ash	0.53±0.11	
pH value (10% aq. Suspension)	6.37±0.27	
Extractive values	Cold extraction	Hot extraction
Hexane	0.78±0.12	0.75±0.09
Chloroform	12.34±0.21	15.98±0.13
Ethyl acetate	7.15±0.11	7.95±0.11
Acetone	6.98±0.17	6.83±0.19
Methanol	17.92±0.31	18.32±0.27
Ethanol	10.65±0.31	10.32±0.33
Water	9.34±0.29	9.78±0.32

Values are expressed as Mean±SD

be skipped during extraction and analysis. All five extracts (hexane, chloroform, ethyl acetate, methanol, and water) were analysed using LC-MS/MS.

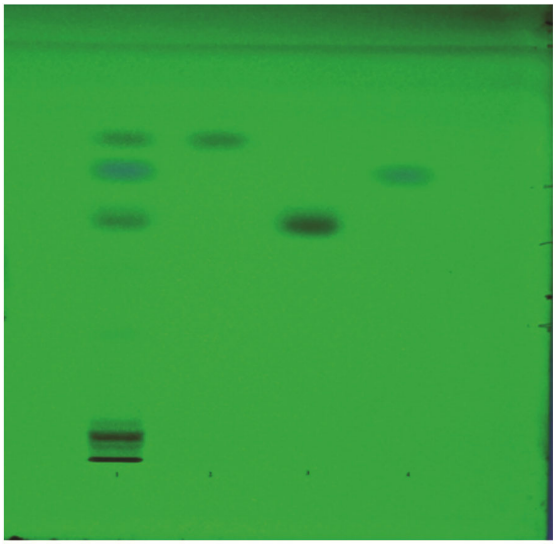

Fig. 5 — HPTLC profiles visualised at 254 nm for *P. cerasoids* heartwood methanol extract for the qualitative detection of flavonoids (Track 1, 2, 3 and 4 are methanol extract (PC-M), biochanin A, genistein and sakuranetin respectively).

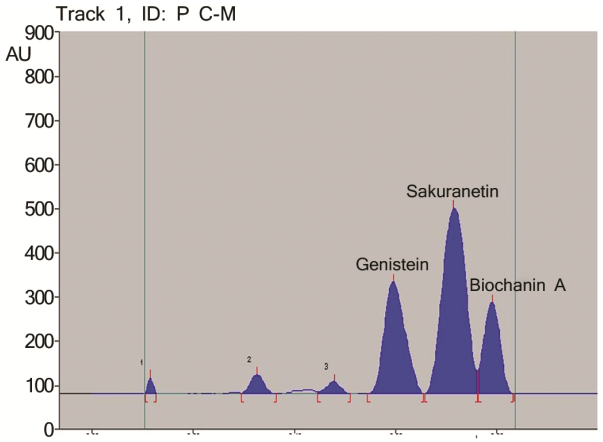

Fig. 6 — HPTLC densitogram of *P. cerasoids* heartwood methanol extract.

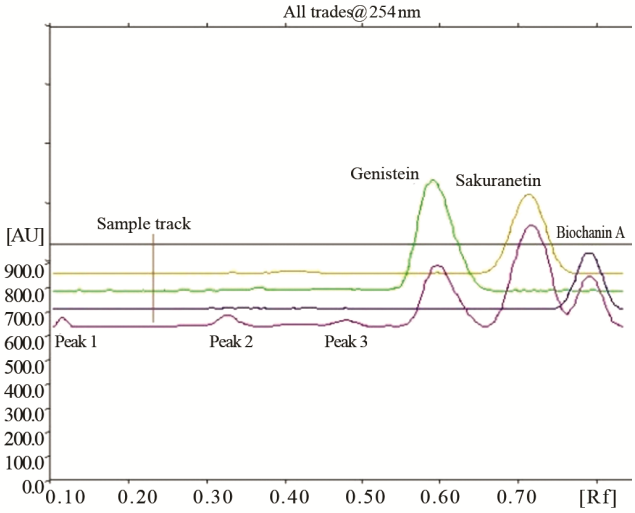

Fig. 7 — Comparative 3D chromatogram of sample and standards.

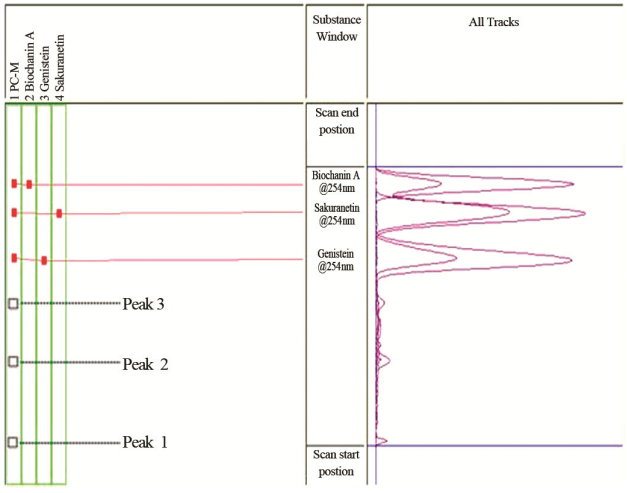

Fig. 8 — Qualitative evaluation of standards and sample tracks by HPTLC.

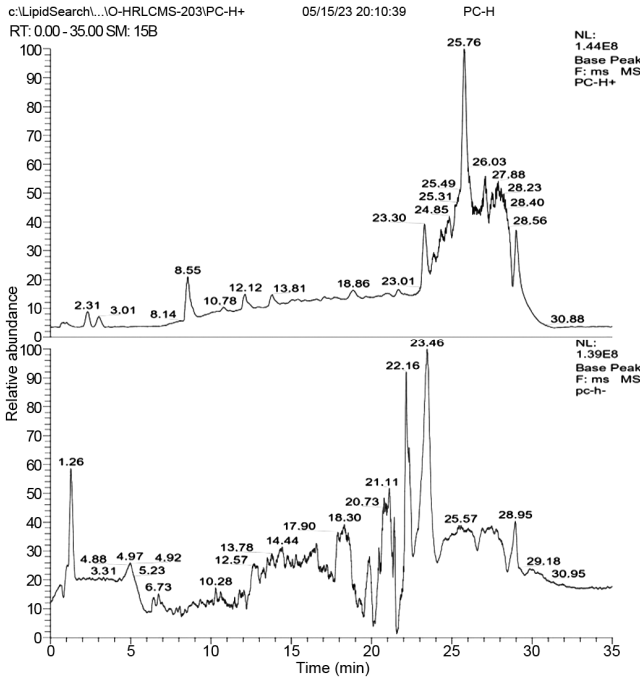

Fig. 9 — LC-MS chromatograms of hexane extract (positive and negative ionisation).

Likewise, LC-MS/MS orbitrap might not detect all the compounds. Still, it is a rapid and effective method with high selectivity and sensitivity. To get the maximum identification, sample preparation and appropriate ionisation mode (positive and negative) were taken into account to detect the maximum number of compounds by this method. In the present investigation we found a number of phytocompounds which were not reported earlier[41]. Compounds like Genistein, Biochanin Naringenin are major among them. To confirm their presence, we did HPTLC profiling with these compomunds. The qualitative presence of these phytocompounds were attributed by chromatographic profiling.

Screening and qualitative identification of secondary metabolites in different solvent extracts were executed by comparing the reported data using MZ cloud software. Images of all the extracts' positive and negative ESI chromatograms are given in Fig. 9-13.

Many compounds were found in LC-MS/MS analysis. Flavonoids were a major of them. Flavonoids, namely, Genistein, Sakuranetin, Biochanin-A, Naringenin, Taxifolin, Prunin, Formononetin, and Eriodictyol, are major. A comprehensive list of metabolites present in different extracts was tabulated in Table 2. The most remarkable observation is that the methanol extract is the most flavonoid-rich fraction

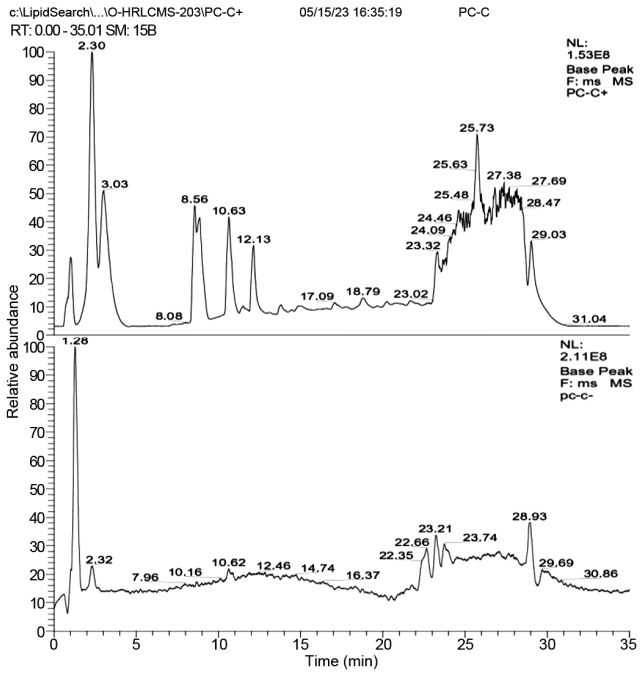

Fig. 10 — LC-MS chromatograms of chloroform extract (positive and negative ionisation).

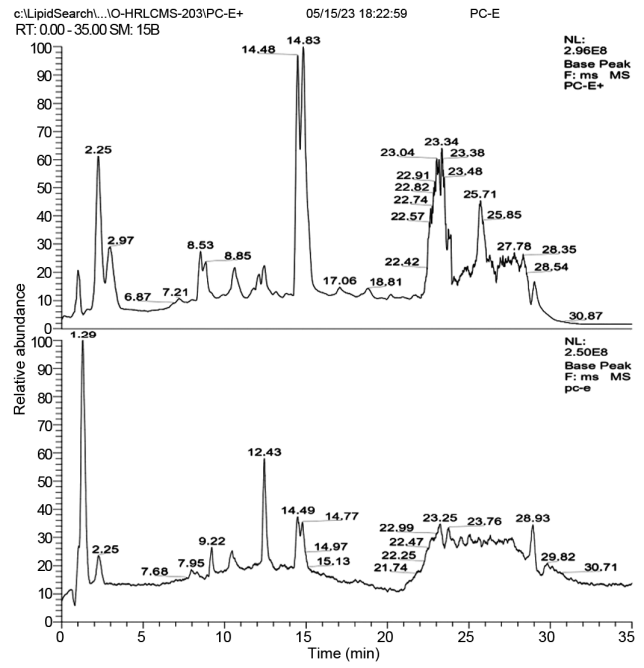

Fig. 11 — LC-MS chromatograms of ethyl acetate extract (positive and negative ionisation).

compared to other extracts. At the same time, the hexane and chloroform were found to have poor extractability. Ethyl acetate showed moderate efficiency in extraction. Although the most eco-friendly solvent in nature, water was proven to be a poor agent in extracting flavonoids from heartwood.

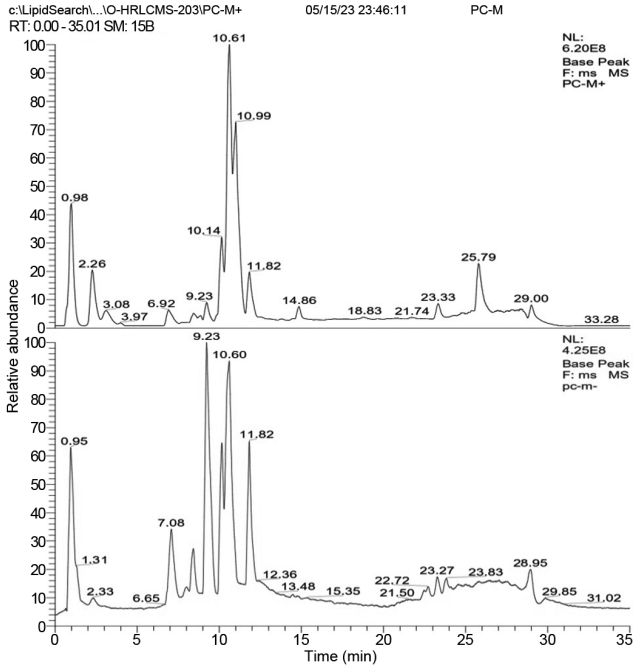

Fig. 12 — LC-MS chromatograms of methanol extract (positive and negative ionisation).

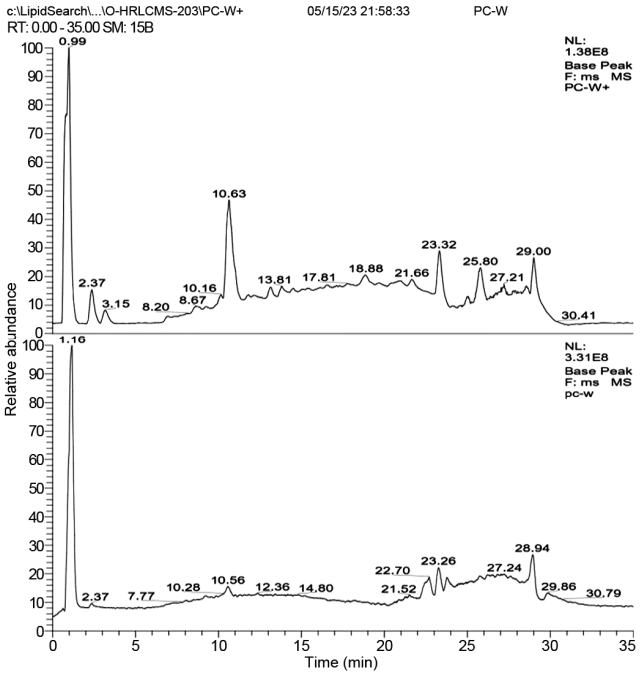

Fig. 13 — LC-MS chromatograms of water extract (positive and negative ionisation).

Among other secondary metabolites, the presence of glycosides, amino acids, derivatives of amino acids, and curcuminoids was detected by LC-MS/MS. Therefore, it may be clearly stated that *P. cerasoids* heartwood, which is a flavonoid-rich plant part, can efficiently be extracted from none other than methanol.

Table 2 — Presence of secondary metabolites identified in different extracts of *P. cerasoids* heartwood

Extracting solvent	Phytocompound identified in LC-MS/MS
Hexane	Betaine, Choline, *cis,cis*-muconic acid, Urocanic acid
Chloroform	Betaine, Caprolactum, Choline, Ethylmalonic acid, Sakuranetin
Ethyl acetate	Apigetrin, Arecoline, Astilbin, Betaine, Catechin, Choline, Eriodictyol, Ethylmalonic acid, Formononetin, Naringenin, Pipecolic acid, Taxifolin, Urocanic acid
Methanol	Apigetrin, Astilbin, Betaine, Biochanin A, Caprolactum, Catechin, Choline, Coumarin, Eriodictyol, Ethylmalonicacid, Formononetin, Genistein, Glycitein, Hematoxylin, Naringenin, Phloretin, Pipecolic acid, Prunin, Quercetin, Rutin, Sakuranetin, Taxifolin, Trifolin
Water	Apigetrin, Astilbin, Betaine, Catechin, Choline, Eriodictyol, Ethylmalonic acid, Genistein, Naringenin, Sakuranetin, Urocanic acid

Names of the phytocompounds are given in alphabetical order, not as per the quantities available in extracts.

Conclusion

The present investigation furnished a set of qualitative and quantitative phyto-pharmacognostic parameters. The data set may be found as a blueprint of this plant part. The outcome may contribute significantly in many aspects. Pharmacognostical characterisation, which is considered to be the most fundamental stepping stone in natural product research, can be a guide in the identification of the right plant. The output of HPTLC fingerprinting contributes to the next step of authentication of the right species. The LC-MS/MS data have given a complete picture of the secondary metabolites present in the plant part that contribute to the medicinal values of a plant. In addition, the variations in the composition of different extracts may be a clue in developing phytopharmaceuticals that align with efficient extractability. Hence, the data set can serve as diagnostic tools for establishing quality standards, authentication, and identifying the medicinally important plant. This helps compile a suitable monograph of this plant and develop pharmaceuticals from natural sources.

Conflict of interest

Authors of the present work declare that there are no conflict of interest associated with this article.

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