Comparative secondary metabolite expression in callus cultures and mother plant in Barleria prionitis L.

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Research Article

Keywords: Active principles, antioxidants, flavonoids, in vitro, iridoid glycoside, phenolics

Posted Date: May 23rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2794569/v1

Abstract

The present study is the first report on the quantitative determination of secondary metabolites in callus cultures of *B. prionitis* L, an important medicinal plant. We examined the stem-derived and leaf-derived callus for their antioxidant potential and accumulation of phenolics, flavonoids, and active principles viz. shanzhiside methyl ester (SME) and squalene and compared these parameters vis a vis mother plant counterpart. Four solvents viz. methanol, ethanol, acetone, and distilled water (aqueous) extracts were prepared for analysis. Callus cultures not only preserved the metabolite profile of the mother plant but also displayed their elevated levels. Leaf-derived callus surpassed stem-derived callus in most of the parameters studied. The highest phenolic content (21.46 mg GAE g^{-1} FW) and flavonoid content (24.58 mg of RE g^{-1} FW) was observed in methanol extract of leaf-derived callus, representing a 3-fold and 2-fold increase over mother plant leaf, respectively. Antioxidant capacity based on FRAP and DPPH assay was highest in methanol extract of leaf-derived callus (7-fold and 3-fold increase over mother plant, respectively) while ABTS assay showed the highest activity (122-fold increase) in acetone extract of leaf-derived callus. HPTLC analysis revealed an enhanced concentration of squalene (10-fold) and SME (2.3-fold) in acetone and methanol extract of leaf-derived callus, respectively, compared to mother explants. Results of RP-HPLC for phenolics showed the highest gallic acid content (99-fold increase) in ethanol extract of stem-derived callus whereas catechol was maximum (37- fold increase**)** in aqueous extract of leaf-derived callus. These findings suggest that callus cultures of *B. prionitis* can be a potential source of active metabolites. Further, cell suspension cultures can be established from the callus which could be an avenue for the large-scale production of bioactive compounds.

Key Message

Callus cultures of *B. prionitis* L. showed amplified antioxidant activity and accumulation of phenolics, flavonoids, squalene and shanzhiside methyl ester compared to the mother plant.

Introduction

The capacity of plant cells, tissue, and organ cultures to produce valuable biochemical compounds has been recognized almost since the inception of plant biotechnology (Chatterjee and Ghosh 2020). Comparative phytochemical evaluation of *in vitro* cultures with the mother plant is necessary to ensure their authenticity in terms of chemical composition. In addition, it also provides a basis for quality assurance (Khan et al. 2021) and the tissue cultures with quality better than the mother plant can thus be recommended for scaling-up production of useful compounds. Research reports focused on such comparative analyses in medicinal plant species are available and some of them revealed synthesis of certain phytochemicals at a level higher than *in vivo* plants (Riahi et al. 2022).

Barleria prionitis L. is a medicinal shrub belonging to the family Acanthaceae. It is used for the treatment of various conditions like toothache, bleeding gums, whooping cough, inflammation, stiffness of limbs, enlargement of the scrotum and sciatica (Gangaram et al. 2022). Its important active principles are shanzhiside methyl ester (SME), barlerin, and squalene apart from phenolics and flavonoids. SME is an iridoid glycoside that has been reported to show various bioactivities, such as anti-inflammatory, anti-depressant, anti-diabetic, antioxidant, and neuroprotective effects (Ghule et al. 2012). Some of the possible mechanisms of action of SME include inhibiting the production of pro-inflammatory cytokines, stimulating the expression of β-endorphin in spinal microglia, modulating the activity of monoamine oxidase and serotonin transporter, enhancing the insulin signaling pathway, scavenging free radicals, and protecting against neuronal damage (Ghule et al. 2020; Sun et al. 2022).

Squalene is a triterpene that is widely distributed in nature and is an intermediate in the cholesterol biosynthesis pathway. It has been reported to exhibit various biological activities, such as antioxidant, anticancer, antidiabetic, anti-inflammatory, neuroprotective, skin hydrating and emollient effects (Lou-Bonafonte et al. 2018). It has multiple industrial applications in different fields such as pharmaceuticals, cosmetics, food supplements and biofuels (Lozano-Grande et al. 2018). Both SME (Ghule et al. 2012) and squalene (Nidhi et al. 2013) have been quantified and isolated in *B. prionitis* using techniques like HPTLC, flash chromatography and GC-MS and structure of these compounds were elucidated with the aid of extensive NMR spectral studies.

The tremendous medicinal potential of *B. prionitis* demands its use as a prospective drug resource to treat many ailments (Farrukh 2022) and utilizing its tissue cultures to extract candidate active compounds would be an ideal choice. A literature survey showed that few attempts have been made toward tissue culture of *B. prionitis* L. (Lone et al. 2011; Kumar and Rani 2016; Singh et al. 2015; Premjet et al. 2010; Shukla et al. 2011; Kumari et al. 2013) but none of these undertook phytochemical characterization of tissue cultures except one report on qualitative analysis of secondary metabolites in callus cultures (Kumari et al. 2013).

Callus is produced because of the dedifferentiation of cells in the explant. It retains the total genetic information of the mother plant and therefore demonstrates biochemical totipotency. Callus cultures have been considered for the incessant production of bioactive metabolites for use in the pharmaceutical, food, and cosmetic industries (Effert 2019). Further, callus cultures can be used to produce cell suspension cultures which can be cultivated in bioreactors to upscale the production.

To exploit tissue cultures for industrial use, it is imperative to undertake their extensive phytochemical characterization. The literature indicated a huge gap in phytochemical studies in tissue cultures of *B. prionitis* L. Therefore, the present investigation aimed to evaluate the antioxidant capacity and contents of phenolics, flavonoids, shanzhiside methyl ester (SME) and squalene in leaf-derived and stem-derived callus cultures in comparison to the mother plant leaf and stem. To the best of our knowledge, this is the first report of its kind in *B. prionitis* L. tissue cultures.

Material and Methods

Plant Material and callus culture

The plants of *B. prionitis* L. were collected from Thane in Maharashtra, India and were authenticated at the Botanical Survey of India, Pune (voucher specimen No. DYP01TH). The plants were maintained in the greenhouse at the School of Biotechnology and Bioinformatics, D. Y. Patil Deemed To Be University, CBD Belapur, to serve as an explant source.

Callus was established from stem and leaf explants. Internodal segments from stems were washed under running tap water and surface sterilized by pre-treatment with sodium hypochlorite (1% active chlorine; v/v) for 7 min, 0.1% mercuric chloride (w/v) for 10 min followed by thorough washing with sterile distilled water and inoculated on callus induction medium i.e. MS medium (Murashige and Skoog, 1962) containing 0.5 mg l^{-1} Naphthalene acetic acid, 0.5 mg l^{-1} 6-benzyl aminopurine and 300 mg l^{-1} ascorbic acid. Callus obtained was multiplied by repeated subculture (four cycles) at an interval of 25 days on the callus induction medium containing 200 mg l^{-1} ascorbic acid, 100 mg l^{-1} polyvinylpyrrolidone and 20 mg l^{-1} citric acid (Ranade 2022).

Leaf explants were surface sterilized by pre-treatment with sodium hypochlorite (1% active chlorine; v/v) for 7 min, 0.1% mercuric chloride (w/v) for 8 min followed by thorough washing with sterile distilled water and inoculated on MS medium supplemented with 1.0 mg l^{-1} 2,4-Dichlorophenoxy acetic acid, 0.5 mg l^{-1} 6-benzyl aminopurine, 300 mg l^{-1} ascorbic acid (callus induction medium). Callus obtained was multiplied by repeated subculture (three cycles) at an interval of 25 days on the callus induction medium containing 200 mg l^{-1} ascorbic acid, 100 mg l^{-1} polyvinylpyrrolidone and 20 mg l^{-1} citric acid (Ranade 2022). The multiplication phase (after the last subculture) callus was used as the experimental material (Fig. 1)

Phytochemical studies

Preparation of extract

Fresh leaf-derived and stem-derived calli, as well as fresh leaves and stems of the mother plant, were ground in liquid nitrogen followed by extraction in four different solvents i.e. ethanol, methanol, acetone and distilled water (aqueous). The sample to solvent ratio was 1:10 w/v (5 g of crushed fresh sample in 50 ml of solvent). The resulting mixture was kept on a shaker overnight (100 rpm) for 10 hrs to facilitate better extraction. The insoluble material was removed by centrifugation at 4000 x g for 15 min (Superspin R-V/Fa, Plasto crafts, India) and the extracts were concentrated by evaporation using rotavapor (Rotary Vacuuma 'Digital Bath', Superfit Continental Pvt. Ltd. India) and used for biochemical studies as described below:

Total phenolic content

The total phenolic content (TPC) was estimated by the Folin Ciocalteu (FC) method (Supritha and Radha 2018). The reaction mixture consisted of 0.5 ml of sample extract (1g L^{-1}), 2.5 ml 10-fold diluted FC reagent and 2 ml 7.5% sodium carbonate (Na_2CO_3). It was incubated in dark at room temperature for 30 minutes followed by a record of absorbance at 760 nm against a blank using a UV visible spectrophotometer (UV-1700 Pharma Spec, Shimadzu). The total phenolic content was calculated from the standard curve of gallic acid and expressed as mg gallic acid equivalent (GAE) g^{-1} FW.

Total flavonoid content

The total flavonoid content (TFC) was measured by aluminium chloride colorimetric assay (Akkol et al. 2008). 1 ml of extract (1g L^{-1}) or standard (rutin) was added to 1 ml of 2 %aluminum chloride ($AlCl_3$) and 3 mL (5%) sodium acetate. The mixture was incubated at 20°C for

2.5 h and the absorbance was recorded against the blank at 440 nm using a UV visible spectrophotometer. The total flavonoid content was calculated from the standard curve of rutin and expressed as mg rutin equivalents (RE) g^{-1} FW.

Analysis of antioxidant capacity

Antioxidant potential was measured using DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging assay (FRSA), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay and Ferric ion reducing antioxidant power assay (FRAP) assay as per the protocols described by Jain et al. (2014).

For the DPPH-FRSA assay, ascorbic acid was used as standard. The ability of extracts (0.01to 0.1 mg ml^{-1}) to scavenge DPPH radical was assessed and % DPPH inhibition was calculated using the formula:

DPPH-FRSA (% inhibition) = Abs of control – Abs of sample/ Abs of control *100

Where Abs control is the absorbance of the control (standard) and Abs sample is the absorbance of the test sample (extract).

The scavenging activities of all concentrations of a sample were plotted on the graph and regression analysis was done. Antioxidant activity was expressed as IC50 value (mg ml^{-1}) calculated from the graph.

For the ABTS assay, ascorbic acid was used as standard. The ability of extracts (0.01 to 0.1 mg ml^{-1}) to scavenge ABTS radical was assessed and % ABTS inhibition was calculated using the formula:

ABTS (% inhibition) = Abs of control – Abs of sample/ Abs of control *100

Where Abs control is the absorbance of the control (standard) and Abs sample is the absorbance of the test sample (extract).

The scavenging activities of all concentrations of a sample were plotted on the graph and regression analysis was done. Antioxidant activity was expressed as IC50 value (µg ml^{-1}) calculated from the graph.

For the FRAP assay, antioxidant activity based on ferric ion-reducing ability was calculated using a standard curve of ascorbic acid at 593 nm. The FRAP result was expressed as mg of ascorbic acid equivalent antioxidant capacity g^{-1} FW (mg AEAC.g^{-1} FW).

Quantification of phenolics - HPLC analysis

RP-HPLC (Waters Model 2487 with UV detector) was performed for the quantification of phenolic compounds in callus and mother plant samples. The stationary phase was C18 column (5µM). The standards of gallic acid, catechol, caffeic acid, ferulic acid and p-coumarin were prepared in methanol (1000 ppm) and diluted to 50 ppm. The elution system was composed of 20% methanol, 1% acetic acid and 80% water with a linear gradient scheme and the detector was adjusted at 280 nm (Ranade et al. 2022).

All four solvent extracts (distilled water, ethanol, methanol and acetone) of callus and mother plant samples were used for HPLC. Identification of phenolic compounds was based on the comparison of their retention time with the standard chromatogram of a mixture of pure phenolic compounds. The concentration of phenolic compounds was calculated from the peak area measurement and was expressed as mg g^{-1}.

Quantification of Shanzhiside methyl ester (SME)

A validated HPTLC method for the determination of SME in *Barleria prionitis* was employed (Ghule et al. 2012). HPTLC system CAMAG LINOMAT 5, including Linomat V sample applicator, UV detector, a CAMAG twin-trough TLC chamber, CAMAG TLC Scanner 3, and software WinCATS – [Linomat 5_130806] were used. Aluminum-backed HPTLC plates (10 cm × 20 cm) with 200-nm thickness of silica gel 60 F254 (Merck, Darmstadt, Germany), prewashed with methanol, were used. SME as a marker (200.0 µg ml^{-1}) in methanol was spotted on the TLC plate at concentrations of 400, 600, 800, 1000 and 1200 ng for preparation of the standard curve. For every TLC plate, at least one spot of SME (600 ng) was applied along with the spots of samples. The mobile phase used was chloroform and methanol in the ratio of (8:2). The compound SME was detected at 254 nm and HPTLC chromatograms along with peak data were obtained. The quantity of SME in each sample was calculated from the standard curve and was expressed as µg g^{-1} FW.

Quantification of Squalene

HPTLC characterization of squalene in callus and mother plant was carried out on the HPTLC system as mentioned in the previous point. Squalene (10 µg ml^{-1} in acetone) was spotted on the TLC plate at concentrations of 50, 100, 150, 200 and 250 ng for preparation of the standard curve. For every TLC plate with sample spots, at least one spot of SQ (150 ng) was applied. The mobile phase used was n-hexane.

The TLC plates were air-dried and sprayed with copper sulphate reagent. The TLC plates were then baked in the oven at 120°C for 10 mins for the development of spots. The separation of compounds was visualized under CAMAG UV detector at 550 nm. The standard curve of peak area versus concentration was plotted and linear regression analysis was carried out. The quantity of SQ in each sample was calculated from the standard curve and was expressed as µg g^{-1} FW.

Statistical Analysis

All the phytochemical parameters were determined for three biological replicates of each sample and the results were expressed as mean values (± standard deviation, SD), calculated using MS Excel 2007 Software (Microsoft Corporation, Redmond, WA, USA). Each parameter was subjected to a one-way analysis of variance (ANOVA) followed by Fischer's LSD test to validate the significance of the difference between means using GraphPad Prism 5.0.

Results and Discussion

Callus cultures of *B. prionitis* L. are known to express secondary metabolites. The present study encompasses a quantitative comparison of useful metabolites and antioxidant efficiency between the callus and the mother plant. Analysis of variance displayed significant ($p \leq 0.05$) differences between them regarding metabolite levels.

Total phenolic content (TPC)

Antioxidant activity is mainly attributed to the presence of phenolics and flavonoids. In the present study, callus derived from both leaf and stem explants showed higher TPC (2.5-3-fold) compared to the respective mother plant part in all the solvent extracts, and the contents in leaf and leaf-derived callus were significantly higher than the stem and stem-derived callus. These findings are supported by previous reports (Jaiswal et al. 2010; Sharma et al. 2014) where the leaf of *B. prionitis* L. was found to be a better source of phenolic compounds than the stem under *in vivo* conditions. The highest phenolic content was observed in the methanol extract of leaf-derived callus (21.46 ± 0.04 mg GAE g^{-1}), representing a 3-fold increase over the mother plant leaf (Table 1). Similar to our observations, higher TPC in leaf-derived callus than the leaf of the mother plant has also been reported in *L. umbellata* (Govindaraju et al. 2019). In our study, TPC in the remaining solvents extracts of leaf-derived callus was not significantly different indicating similar extraction capacity of the solvents for total phenolics while Govindaraju et al. (2019) reported that methanol had the highest extraction capacity for phenolics leaf-derived callus of *L. umbellata.*

Table 1
Total phenolic contents and Total flavonoid contents in callus and mother plant of *B. prionitis* L.

Extracts	Total phenolic contents (mg GAE g^{-1} FW)				Total flavonoid contents (mg RE g^{-1} FW)			
	Mother plant		Callus		Mother plant		Callus	
	Leaf	Stem	Leaf derived	Stem derived	Leaf	Stem	Leaf derived	Stem derived
Ethanol	8.26±0.08^{d}	4.96±0.05^{e}	19.07±0.05^{a}	12.33±0.22^{b}	16.54±0.13^{c}	10.12±0.05^{f}	14.90±0.06^{d}	11.32±0.10^{f}
Methanol	7.93±0.12^{d}	5.62±0.03^{e}	21.47±0.05 ^{a}	14.09±0.50^{b}	13.51±0.22^{e}	7.59±0.02^{g}	24.58±0.05 ^{a}	19.34±0.15^{b}
Acetone	9.92±0.09^{c}	7.36±0.08^{d}	20.21±1.10 ^{a}	13.61±0.45^{b}	16.21±0.06^{c}	11.23±0.08^{f}	13.36±0.10^{e}	11.83±0.5^{f}
DW	5.44±1.22^{e}	3.55±1.02^{f}	19.37±0.13 ^{a}	9.45±1.00^{c}	5.71±0.11^{h}	4.26±0.05^{i}	11.51±0.10^{f}	10.53±0.21^{f}

Values are expressed as mean ± SD (n = 3) and the means followed by different letters differ significantly at $p < 0.05$, Fisher's LSD test. GAE-Gallic Acid Equivalent, RE-Rutin Equivalent, DW-Distilled Water, FW-Fresh Weight

Total flavonoid content (TFC)

In the present study, TFC was higher in both leaf and stem-derived calli over respective mother plant parts in methanol and aqueous extracts only. Similar as well as contrasting observations have been reported by other workers like lower TFC contents in stem-derived callus compared to the mother plant in *S. kakudensis* (Manivannan et al. 2015) while higher TFC in leaf-derived callus than the leaf of mother plant in *L. pumila* var. alata (Najhah et al. 2021). These observations indicate that the concentrations are dependent on plant species, plant part and solvent type.

The highest TFC was observed in methanol extract of leaf-derived callus (24.58 mg RE g^{-1}), representing a 2-fold increase over the mother plant leaf in *B. prionitis* (Table 1). Contrary to this, Moteriya et al. (2014a) reported acetone as the best solvent for the extraction of total flavonoids. Leaf-derived callus showed significantly higher TFC compared to stem callus in all the solvent extracts except distilled water in our study. These results were supported by observations in *Maytenus emarginata* (Moteriya et al. 2014a) and *Gloriosa superba* L. (Moteriya et al. 2014b), where leaf-derived callus was richer in flavonoids than stem callus.

Antioxidant activity

In the present study, antioxidant activity based on FRAP assay was significantly higher in both the calli compared to the respective mother plant part in all the solvent extracts. (Table 2). The highest FRAP values were recorded in methanol extract of leaf-derived callus (253.77 mg AAE g^{-1}), representing an increase of 7- fold over the mother plant leaf (Table 2). Similar results were reported by Bahorun et al. (2002) where callus cultures of *C. fistula* initiated from young leaves showed high FRAP activity. In the present study, the leaf and stem of the mother plant showed almost similar FRAP values, but the callus obtained from these two explants showed significantly different activity in all solvent extracts, with the higher activities seen in leaf-derived callus over stem-derived callus.

Table 2
Antioxidant potential of callus culture and mother plant of *B. prionitis* L.

Antioxidant Assay	Extracts	Mother Plant		Callus	
		Stem	Leaf	Stem-derived	Leaf-derived
FRAP activity (mg AAE g^{-1})	Ethanol	32.62 ± 0.09^{e}	38.14 ± 0.51^{d}	45.30 ± 0.11^{c}	156.58 ± 0.03^{b}
	Methanol	36.78 ± 0.02^{d}	34.10 ± 0.19^{e}	157.94 ± 0.05^{b}	253.77 ± 0.33^{a}
	Acetone	32.82 ± 0.20^{e}	36.67 ± 0.10^{d}	43.45 ± 0.05^{c}	137.31 ± 0.46^{b}
	DW	28.87 ± 0.34^{f}	27.82 ± 0.10^{f}	37.62 ± 0.17^{d}	46.78 ± 0.05^{c}
DPPH radical scavenging activity (IC50; mg ml^{-1})	Ethanol	35.21 ± 0.11^{d}	33.59 ± 0.17^{d}	39.64 ± 0.71^{e}	21.81 ± 0.01^{b}
	Methanol	21.47 ± 0.20^{b}	46.22 ± 0.23^{f}	20.58 ± 1.21^{b}	16.55 ± 0.05^{a}
	Acetone	34.65 ± 0.44^{d}	26.98 ± 0.09^{c}	29.11 ± 0.56^{d}	20.96 ± 0.16^{b}
	DW	69.54 ± 0.50^{i}	61.54 ± 0.15^{g}	105.07 ± 0.29^{i}	76.85 ± 1.10^{h}
ABTS radical scavenging activity (IC50; µg ml^{-1})	Ethanol	453.04 ± 1.03^{f}	691.12 ± 0.09^{h}	487.92 ± 0.45^{g}	299.43 ± 0.45^{d}
	Methanol	889.95 ± 1.11^{j}	814.82 ± 0.11^{i}	367.34 ± 1.19^{e}	249.21 ± 0.33^{c}
	Acetone	4.62 ± 0.05^{b}	366.97 ± 0.16^{e}	4.61 ± 0.20^{b}	3.08 ± 0.03^{a}
	DW	5954.05 ± 0.61^{n}	5118.99 ± 0.07^{m}	1144.99 ± 0.50^{k}	1459.51 ± 0.06^{l}

Values are expressed as mean ± SD (n = 3) and the means followed by different letters differ significantly at $p < 0.05$, Fisher's LSD test. DW-Distilled water, AAE-Ascorbic acid equivalent

In the present study, as compared to the mother plant, stem-derived callus (methanol and acetone extract) and leaf-derived callus (all except aqueous extract) showed higher DPPH radical scavenging activity with the highest activity of 16.55mg ml^{-1} IC 50 (3-fold increase) seen in methanol extract of leaf-derived callus (Table 2). Similar observations on higher DPPH activity in leaf-derived callus over the leaf of the mother plant have been recorded in *L. umbellata* (Govindaraju et al. 2019)d *pumila* var. alata (Najhah et al. 2021) Jaiswal et al. (2010) and Sharma et al. (2014) reported higher DPPH radical scavenging activity in the leaf than stem in *B. prionitis* L. which is in line with our observations.

In the present study, both leaf and stem- derived calli showed higher (3 to 5-fold) ABTS scavenging activity compared to the mother plant in most of the extracts and the highest activity (122-fold increase) was recorded in the acetone extract of leaf callus (Table 2). Contrastingly, field-grown leaves showed three times more ABTS activity than leaf-derived calli in blueberry and blackberry (Kolarević et al. 2021). Chavan et al. (2011) found acetone to exhibit the best extraction capacity for ABTS scavenging antioxidants in *B. prionitis*.

All three antioxidant assays showed higher antioxidant activity in callus cultures over the mother plant and this could be attributed to the fact that *in vitro* conditions are experienced as stress by the cultured tissues and in order to combat stress the tissues accumulate antioxidants (Najhah et al. 2021). However, the comparison of the three assays revealed that they did not show identical trends in antioxidant activities with regard to solvent and plant part types. These differences could be because the three methods have different principles and mechanisms to measure antioxidant potential and due to differences in the type and concentrations of antioxidants present in the samples. It is recognized that antioxidants differ in their reactivity with DPPH, ABTS and transition metal ions while sometimes a mixture of polyphenols can produce a harmonious effect on DPPH and FRAP (Khorsidi and Nijavan 2006).

Quantification of phenolics

RP-HPLC for quantification of five phenolic compounds i.e. Gallic acid, Catechol, Caffeic acid, p-Coumaric acid and Ferulic acid was performed for all the extracts. It was found that only gallic acid and catechol were detected in a few samples (Table 3). Stem-derived callus showed the presence of gallic acid in all the extracts and the highest content of 99.08 µg g^{-1} FW (99-fold) was recorded in ethanol extract whereas it was not detected in its mother plant counterpart (Fig. 2). Leaf-derived callus showed the presence of gallic acid only in aqueous extract and it was 10-fold higher than the mother plant leaf. The aqueous extracts of the leaf (11.16 µg g^{-1} FW) and stem-derived (10.0 µg g^{-1} FW) callus showed similar gallic acid contents. Catechol was detected only in aqueous extracts of the mother plant stem (5.22 µg g^{-1} FW) and leaf-derived callus (37.43 µg g^{-1} FW). The absence of other phenolics in the samples could be because their synthesis was not within the detection limits.

Table 3
RP-HPLC-based quantification of phenolic compounds in callus culture and mother plant of *B. prionitis* L.

Extract	Gallic acid (µg g^{-1})	Catechol (µg g^{-1})	Caffeic acid (µg g^{-1})	p-Coumaric acid (µg g^{-1})	Ferulic acid (µg g^{-1})
MPLE	ND	ND	ND	ND	ND
MPLM	4.24±0.04^{c}	ND	ND	ND	ND
MPLDW	1.92±0.08^{d}	ND	ND	ND	ND
MPSE	ND	ND	ND	ND	ND
MPSM	ND	ND	ND	ND	ND
MPSDW	2.13±0.10^{d}	5.22±0.56^{b}	ND	ND	ND
LCE	ND	ND	ND	ND	ND
LCM	ND	ND	ND	ND	ND
LCDW	11.16±0.05^{b}	37.43±0.21^{a}	ND	ND	ND
SCE	99.08±0.16^{a}	ND	ND	ND	ND
SCM	2.40±0.01^{d}	ND	ND	ND	ND
SCDW	10.00±0.11^{b}	ND	ND	ND	ND

*Values are expressed as mean ± SD (n = 3) and the means followed by different letters differ significantly at $p < 0.05$, Fisher's LSD test. MPLE = mother plant leaf ethanol extract;

MPLM = mother plant leaf methanol extract; MPLDW = mother plant leaf distilled water extract; MPSE = mother plant stem ethanol extract; MPSM = mother plant stem methanol

extract; MPSDW = mother plant stem distilled water extract; LCE = leaf callus ethanol extract; LCM = leaf callus methanol extract; LCDW = leaf callus distilled water extract;

SCE = stem callus ethanol extract; SCM = stem callus methanol extract; SCDW = stem callus distilled water extract, ND-not detected

Quantification of Shanzhiside methyl ester (SME) and Squalene (SQ) by HPTLC method

In the present study, SME was detected in both leaf (methanol, acetone and aqueous extract) and stem (methanol and acetone extract) of the mother plant in concentrations ranging from 49.69-182.49 µg g^{-1} FW. SME amounts determined by HPTLC were reported to be 4.91% w/w in whole aerial parts (Ghule et al. 2012) and 2.62% w/w in leaf (Kaur et al. 2014) of *B. prionitis*. Callus derived from both the explants also showed the presence of SME in the concentrations significantly higher than the mother plant counterparts (Table 4) and the highest content of 423.82 µg g^{-1} FW (2.3-fold increase) was recorded in methanol extract of leaf-derived callus (Fig. 3). Contrasting to our results, Premjet et al. (2010) reported the absence of SME in leaf callus of *B. prionitis* L. based on the results of TLC and HPLC. In our study, methanol was found to be the best solvent for the extraction of SME in all samples and this agrees with the observations reported by Ghule et al. (2012) and Kaur et al. (2014).

Table 4
HPTLC-based quantification of Shanzhiside Methyl Ester (SME)and Squalene in callus culture and mother plant of *B. prionitis*

Extracts	Concentration of SME (µg g^{-1} FW)				Concentration of Squalene (µg g^{-1} FW)			
	Mother plant		Callus		Mother plant		Callus	
	Leaf	Stem	Leaf derived	Stem derived	Leaf	Stem	Leaf derived	Stem derived
Ethanol	-	-	152.66±0.14^{f}	286.95±0.02^{c}	0.33±0.02^{f}	0.65±0.10^{e}	5.11±0.12^{c}	0.30±0.07^{e}
Methanol	182.49±0.45^{e}	156.54±0.06^{f}	423.82±0.11^{a}	348.18±0.05^{b}	-	1.83±0.03^{d}	10.74±0.12^{b}	0.62±0.07^{e}
Acetone	62.43±1.22^{h}	127.84±0.66^{g}	182.89±0.10^{e}	259.37±0.16^{d}	1.40±0.05^{d}	4.04±0.05^{c}	14.23±0.21^{a}	1.15±0.00^{d}
DW	49.69±0.31^{i}	-	58.36±0.07^{h}	-	NQ	NQ	NQ	NQ

Values are expressed as mean ± SD (n = 3) and the means followed by different letters differ significantly at $p < 0.05$, Fisher's LSD test. NQ-not quantified. FW-Fresh weight

Squalene is present in *B. prionitis* L. roots as revealed by GC-MS studies (Nidhi et al. 2013). In the present investigation, HPTLC analysis revealed the presence of squalene in all mother plant and callus samples except methanol extracts of the mother plant leaf. Its quantity was higher (1.8 to 2.8- fold) in the stem than in the leaf of the mother plant while a reverse pattern was noticed in the respective callus (Table 4). Out of the two calli, only leaf-derived callus showed more squalene than the mother plant leaf in all the extracts and the highest content of 14.23 µg g^{-1} FW (10-fold increase) was obtained in acetone extract (Fig. 3). Popa et al. (2015) also suggested that solvents like acetone are preferable for extraction of squalene due to its low polarity. Like our results, a 2.3-fold increased production of squalene was observed in leaf-derived callus than in leaves of field-grown plants of *Nilgirianthus ciliates* (Rameshkumar et al. 2018). In leaf-derived callus, antioxidant activity corresponded with the squalene concentration in all the extracts indicating the contribution of squalene as an antioxidant in the present study.

Conclusion

Our investigations confirmed the presence of useful metabolites of *B. prionitis* in its callus cultures. Further, callus cultures exhibited higher antioxidant activity and accumulation of phenolics, flavonoids, squalene and shanzhiside methyl ester compared to the mother plant with a remarkable rise in squalene content by 10-fold in leaf-derived callus. Leaf-derived callus surpassed stem-derived callus in all the studied parameters except catechol content. Methanol showed the highest extraction capacity for the majority of the metabolites. These findings suggest that leaf-derived callus cultures can be explored for viable production of active principles of *B. prionitis* L.

Declarations

Funding: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Authors Contribution Statement: N. Josh-conception of the idea, experiment planning, interpretation of results and manuscript preparation, R. Ranade- conduction of experiments, and interpretation of results S. Kudale- interpretation of results, data analysis, manuscript review.

Competing Interests: The authors declare that they have no conflict of interest.

Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author at a reasonable request.

Acknowledgments: Authors are thankful to Dr. B.V. Ghule, Institute of Pharmaceutical Education and Research, Wardha, Maharashtra, India for providing guidance and laboratory facilities and SME standards for HPTLC. Support and resources provided by Dr. Debjani Dasgupta, Director, School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Navi Mumbai are gratefully acknowledged.

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Figures

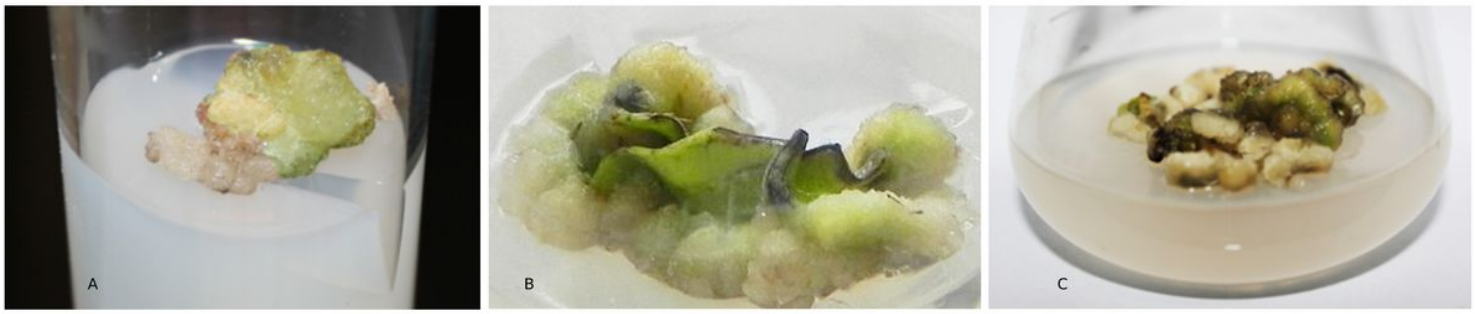

Figure 1

In vitro callus cultures of *B. prionitis* L. (A) Callus induction from stem explant, (B) Callus induction from leaf explant, (C) Multiplication phase callus culture

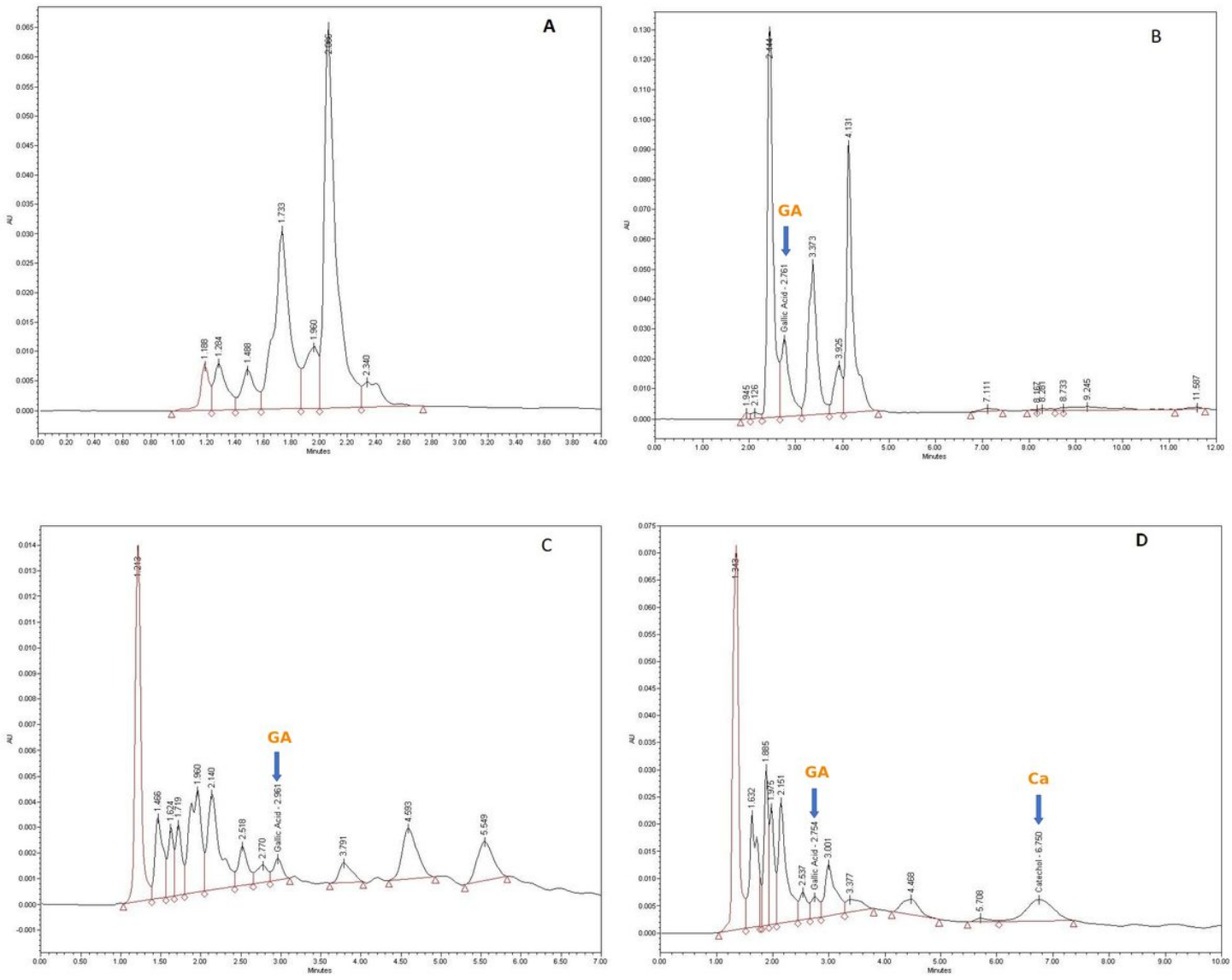

Figure 2

RP-HPLC chromatogram of phenolics - Ethanol extract of mother plant stem (A) and stem-derived callus (B) showing the absence and presence of gallic acid (GA), respectively; Aqueous extract of mother plant leaf (C) and leaf-derived callus (D) showing the absence and presence of catechol (Ca), respectively.

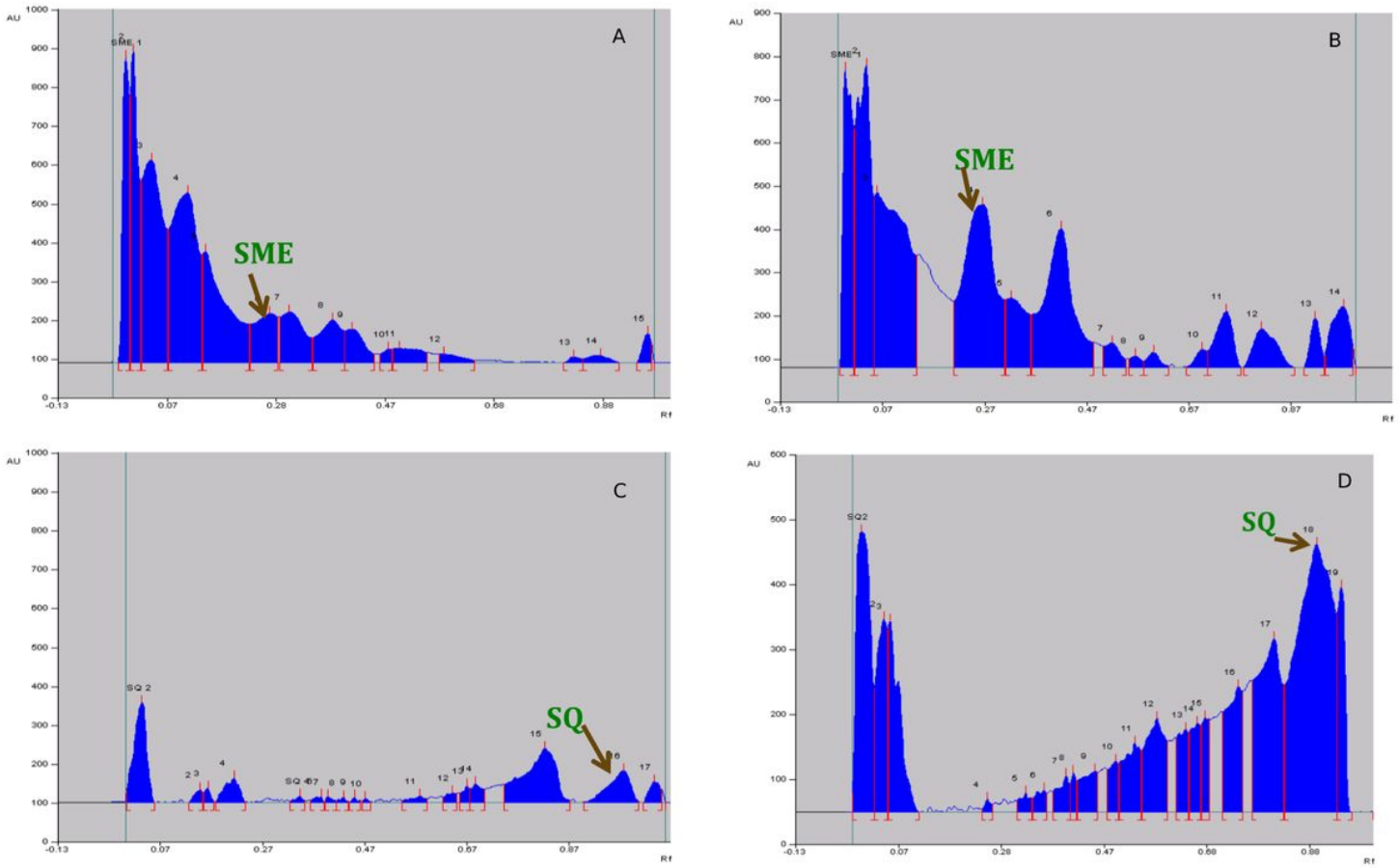

Figure 3

HPTLC chromatogram - Shanzhiside methyl ester (SME) in methanol extract of mother plant leaf (A) and leaf-derived callus (B); Squalene (SQ) in acetone extract of mother plant leaf (C) and leaf-derived callus (D)