Journal of Food Measurement and Characterization (2023) 17:1343–1353
https://doi.org/10.1007/s11694-022-01695-6

ORIGINAL PAPER

Optimization of extraction of bioactive phenolics and their antioxidant potential from callus and leaf extracts of *Citrus sinensis* (L.) Osbeck, *C. reticulata* Blanco and *C. maxima* (Burm.) Merr.

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Received: 27 June 2022 / Accepted: 1 November 2022 / Published online: 22 November 2022

Abstract
The present study was conducted with an objective of optimizing extraction conditions for achieving maximum recovery of total phenolic content (TPC), total flavonoid content (TFC) and antioxidant potential in terms of radical scavenging activity (RSA) from ethanolic extracts of callus cultures and leaf tissues of three citrus species i.e. sweet orange (*Citrus sinensis* (L.) Osbeck), mandarin (*Citrus reticulata* Blanco) and pummelo (*Citrus maxima* (Burm.) Merr.). The callus was obtained from the seeds germinated under in vitro conditions. The extracts from callus cultures and leaf tissues were prepared in ethanol solutions (25%, 50% and 75%) by ultrasound-assisted extraction at different temperatures (30, 50 and 70 °C) and extraction times (10, 20 and 30 min). The results revealed that the extraction conditions of ethanol: water ratio of 50% at 50 °C for 30 min were the best in recovering maximum amounts of TPC, TFC and RSA. Analysis of variance was performed to identify various optimization conditions. A correlation analysis was performed to effect the interaction of phenols and flavonoids on antioxidant potential of callus cultures and leaf tissues in three citrus species. It was concluded that callus and leaves of the citrus species possess great potential for their use in production of bioactive compounds.

Keywords *Citrus maxima* (Burm.) Merr. · *Citrus reticulata* Blanco · *Citrus sinensis* (L.) Osbeck · Correlation analysis · Radical scavenging activity · Total phenol content · Total flavonoid content

Introduction

The genus *Citrus* belongs to family Rutaceae which consists of 130 genera classified into seven subfamilies [1]. The species and different cultivars of Rutaceae are being used for commercial production of fruits which provide a wide range of flavor, acidity, sweetness, color and size. Citrus fruits are the world's most widely grown subtropical fruits with annual global production of 143755.6 thousand tonnes in 2019 out of which 71887.8 thousand tones was produced in Asia [2, 3]. Pertaining to frequent intergeneric and interspecific hybridization, bud mutations, vast history of cultivation, and apomixes; a huge amount of genetic variation exists between citrus species [4].Citrus, being an important fruit, is usually consumed fresh and is also widely used as food additive, dietary supplement, and herbal medicine. Citrus is thought to have anti-inflammatory, antimicrobial and antioxidant properties and is believed to be responsible for curing degenerative diseases and cancer [5]. Furthermore, citrus fruits are an imperative source of various bioactive compounds including antioxidants like flavonoids, ascorbic acid and phenolic compounds essential for human nutrition [6]. Being a rich source of phytochemicals, citrus has number of health maintaining properties. These phytochemicals, including macro (sugars and dietetic fibers) and micronutrients (folate, thiamin, niacin, vitamin B_6, riboflavin, pantothenic acid, potassium, calcium, phosphorus, and magnesium), possess unique disease preventing properties [7]. Citrus fruit flavor and its distinct pleasant aroma derived from complex amalgamation of volatile and soluble compounds are the important constituents of human diet [8]. These compounds comprise

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mainly of sugar, organic acid, large number of carotenoids with secondary metabolites [7] (flavonoids, polysaccharides, lignins, fibers, polyphenols, essential oils, etc.) which are surely powerful disease preventing bioactive components for various degenerative diseases. Many studies have been conducted on citrus fruit properties. Total phenols, total flavonoids and single flavonoids have been studied regarding influence of many factors such as harvest date and cultivar [9]; juice processing [10]; citrus genus and species [11, 12].

Antioxidants as additives can stop in vivo radical reactions, which can destroy nucleic acids and proteins [13]. Phenolic compounds, basically flavonoids, have proved to possess a crucial antioxidant activity against these radicals, chiefly based on their structural characteristics [14]. Wang et al. [15] established a linear relationship among antioxidant activity and phenolic compounds of citrus juices, however, differences in antioxidant activity are perhaps due to variable climate, soil, variety of the fruit, and degree of maturation. Flavonoids are also thought to have a role in providing plant resistance against fungi and bacteria and can show resistance to infections by inhibiting and crosslinking microbial enzymes, chelating metals required for enzyme function, and forming a physical barrier [16].

Various biotechnological assays have been used to perform comparative analysis of TPC (Total phenolic content) and TFC (Total flavonoid content) in different plant tissues and callus in *Plectranthus barbatus Andrews* [17], mentha [18], ginger [19], and ephedra [20]. Biotechnological methods, mainly plant tissue culture tools, play a crucial role in the production of these bioactive ingredients. Callus can easily be produced from a totipotent differentiated cell which has the capability to regenerate into a complete plant [21]. Moreover, callus can be produced from any part of the plant at any time of the year [22]. In vitro callus culture has the ability to eliminate diseases while also allowing for the creation of novel cultivars by inducing somaclonal variations [23].The appropriate culture medium and growth conditions are essential for the biosynthesis of plant metabolites [24, 25]. The potential of citrus plants for the production of various bioactive compounds can be better utilized if its callus also possesses similar compounds and imparts various biological effects as there will be no seasonal and location barrier. In order to achieve this, comparison of callus with plant tissues for the production of antioxidant compounds is needed. To the best of our knowledge, comparative analysis of TPC, TFC and antioxidant activity from callus and leaves of sweet orange, pummelo and mandarin has not been explored yet. Therefore, the present study aimed at optimization of extraction conditions of phenolic compounds from callus cultures and leaf tissues of three citrus species *vis-à-vis* sweet orange *(Citrus sinensis* (L.) Osbeck*)*, pummelo (*Citrus maxima* (Burm.) Merr.), and mandarin *(Citrus reticulata* Blanco) on the basis of antioxidant activity, phenolic content and flavonoids.

Materials and methods

Plant material and callus induction

Seeds of sweet orange, mandarin and pummelo were separated from the fruits of plants growing in the orchards of Department of Fruit Science, Punjab Agricultural University, Ludhiana. The seed coats were removed and were surface sterilized with Teepol followed by washing with tap water. The treatment against fungus was given using1% Carbendazim™. The antibacterial treatment was given with 0.1% sterilized mercuric chloride followed by three washings with sterilized water under laminar air flow cabinet. The seedlings were induced from the seeds transferred onto Murashige and Skoog's (MS) medium [26] having 4 mg L^{-1} BAP (6-Benzylaminopurine) and 0.5 mg L^{-1} kin (kinetin). The seeds were allowed to germinate in an incubation room of a commercial tissue culture laboratory. The incubated cultures were used as an explant for the induction of callus. Leaves, roots, internodes, and nodes of the seedlings were cut off and transferred onto fresh MS medium supplemented with 4 mg L^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid) and 0.5 mg L^{-1} Kin (Kinetin) and incubated at 24 ± 2 °C for callus induction.

The embryonic portion of callus and leaves of the seedlings obtained from the three citrus species were used to determine total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity in terms of radical scavenging activity (RSA).

Preparation of callus and leaf extracts

The callus cultures and leaf tissues were subjected to simultaneous drying and surface sterilization by incubating at high temperature (80° C) until the tissues achieved constant weights. The dried tissues were powdered and the extracts from powdered samples were prepared using mixture of ethanol and water (E:W ratio of 25, 50 and75) using ultrasound-assisted extraction (170 W, 42 kHz) at different temperatures (30, 50 and 70 °C) and extraction times (10, 20 and 30 min). A combination of different extraction treatments (1–27) were obtained as shown in Table 1.

The prepared crude extracts were filtered using Whatmann filter paper No. 1 followed by evaporation under reduced pressure. Finally, 1% activated charcoal was used to decolorize the extracts *via* continuous shaking for 10 min and then stored in dark colored bottles at 4 °C until further use.

Table 1 Different extraction conditionsfor preparing callus and leaf tissue extracts of citrus species

Treatment	E:W ratio	Temperature (°C)	Time (min)
1	25	30	10
2	25	30	20
3	25	30	30
4	25	50	10
5	25	50	20
6	25	50	30
7	25	70	10
8	25	70	20
9	25	70	30
10	50	30	10
11	50	30	20
12	50	30	30
13	50	50	10
14	50	50	20
15	50	50	30
16	50	70	10
17	50	70	20
18	50	70	30
19	75	30	10
20	75	30	20
21	75	30	30
22	75	50	10
23	75	50	20
24	75	50	30
25	75	70	10
26	75	70	20
27	75	70	30

Estimation of total phenolic content and total flavonoid content

The TPC of leaf tissue and in vitro callus crude extracts were determined by using Folin–Ciocalteu reagent [27]. About 0.5 mL of each extract was mixed separately with 2.0 mL of 20% sodium carbonate (Na_2CO_3) solution and 1.5 mL of Folin–Ciocalteu reagent. The final volume of 5.0 mL was made by adding distilled water. The solution was incubated at 55 °C for 90 min and absorbance of the developed color was measured at 760 nm using UV visible spectrophotometer(Shimadzu UV-1800).The TPC was calculated as mg gallic acid per gram dry weight (mg gallic acid g^{-1} dw) by constructing a standard curve of gallic acid (1000–5000 µg mL^{-1}).

The TFC of crude extracts was determined using colorimetric method described by Zeng et al. [28].For the estimation,1.0 mL of crude extract was dissolved in 0.3 mL of 5% sodium nitrite ($NaNO_2$) solution. After 6 min, 0.3 mL of 10% aluminium nitrate [$Al(NO_3)_3$] was added and after another 6 min, 4.0 mL of 4% sodium hydroxide (NaOH) was added. The final volume of 10 mL was prepared with distilled water and maintained at room temperature for 15 min. Absorbance was read at 510 nm against reagent blank using UV–visible spectrophotometer (Shimadzu UV-1800). The TFC was expressed as mg rutin equivalent (mg rutin g^{-1} dw).

Determination of antioxidant potential- radical scavenging activity (RSA)

The hydrogen donating or RSA of crude extracts of leaf tissues and callus cultures were determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) radical [29]. To 0.5 mg mL^{-1} sample (prepared in ethanol), 3.0 mL of freshly prepared 200 mM solution of DPPH in methanol was added and stirred vigorously for 15 s. The samples were incubated at 37° C in water bath for 20 min and absorbance was read at 517 nm. Gallic acid and ascorbic acid were used as standard references. The inhibition percentage was calculated as:

$$\%RSA = \frac{Ac_o - Aa_t}{Ac_o} \times 100$$

where Ac_0 is absorbance of control DPPH solution at 0 min and Aa_t is absorbance of sample after 20 min.

Statistical analysis

Different reaction conditions were optimized for extract preparation to get maximum TPC, TFC and RSA from tissue and callus of pummelo, sweet orange and mandarin. The data was taken in triplicates for all the measurements. Analysis of variance at $p \leq 0.05$ level of significance was performed to study the effect of extraction parameters. The principal component analysis (PCA) and correlation analysis were performed using SAS version 9.4 (SAS Institute Inc.)

Results and discussion

For the germination, seeds of sweet orange, pummelo and mandarin were grown on MS medium supplemented with BAP and Kin. The growth of seedlings initiated within 25 days of culturing. The nodal segments (cotyledonary leaves, hypocotyl, and epicotyl) and leaves showed different responses of callus establishment on MS medium supplemented with 2,4-D and Kin. When MS medium supplemented with 2,4-D (1.5 mg L^{-1}) + Kin (0.5 mg L^{-1}) was used for pummelo percent responses of callus induction were 50%, 25% and 25% from cotyledonary leaves, hypocotyl and epicotyls, respectively. For sweet orange, callus

induction response was 33.3% from cotyledonary leaves. For mandarin cotyledonary leaves, hypocotyl, epicotyl and leaves showed 50%, 40%, 50% and 40% responses of callus induction respectively. Calli were not induced from pummelo leaves and hypocotyl, epicotyl and leaves of sweet orange. When 2,4-D concentration was increased to 2.5 mg L^{-1}, an increase in percent response of callus induction was observed for all three citrus species: pummelo (cotyledonary leaves (75%); hypocotyl (58.5%); epicotyl (55%)), sweet orange (cotyledonary leaves (77.78%); hypocotyl (77.78%); epicotyl (76.92%)); mandarin (cotyledonary leaves (55.56%); hypocotyl (75%); epicotyls (70%); leaves (52.63%)). When the concentration of 2,4-D was further increased to 4.5 mg L^{-1}, a reduction in the growth of callus cultures was observed. The germination and establishment of callus on MS media supplemented with 2,4-D and Kin in three citrus species is shown in Fig. 1.Effect of extraction conditions on TPC, TFC and RSA.

Different combinations of extraction treatments were employed to enhance the yields of phenolic compounds from callus cultures and leaf tissue samples. The maximum TPC in the case of pummelo (6.86 ± 0.04 mg gallic acid g^{-1} dw for leaf tissue and 4.32 ± 0.07 mg gallic acid g^{-1} dw for callus), mandarin (10.76 ± 0.03 mg gallic acid g^{-1} dw for leaf tissue and 9.24 ± 0.04 mg gallic acid g^{-1} dw for callus) and sweet orange (9.78 ± 0.03 mg gallic acid g^{-1} dw for leaf tissue and 8.17 ± 0.04 mg gallic acid g^{-1} dw for callus) were obtained for extraction conditions T15 (E:W ratio of 50 at 50 °C for 30 min) as shown in Table 2 which were significantly higher than all other extraction treatments. The TPC in case of pummelo callus (4.8 ± 0.04 mg gallic acid g^{-1} dw) was observed to be maximum in T6 (E:W ratio of 25 at 50 °C for 30 min) which was significantly higher than TPC in case of T15 (4.32 ± 0.07 mg gallic acid g^{-1} dw). The TPC was found to be higher in the case of leaf tissue for all three citrus species as compared to callus cultures.

Similarly, extraction conditions were optimized to achieve higher TFC from leaf tissues and callus cultures. The extractions of E:W ratio of 50 at 50 °C for 30 min (T15) yielded the maximum TFC from both leaves and callus of the three citrus species (Table 3) which were statistically higher than all other extraction treatments at $p < 0.05$ level of significance except in case of TFC for pummelo callus. The highest TFC in pummelo was 3.25 ± 0.03 mg rutin g^{-1} dw in leaf tissueand 2.6 ± 0.06 mg rutin g^{-1} dw in callus; in case of sweet orange, 5.94 ± 0.07 mg rutin g^{-1} dw in leaf tissue and 4.92 ± 0.02 mg rutin g^{-1} dw in callus; and in case of mandarin, 6.56 ± 0.05 mg rutin g^{-1} dw in leaf tissue and 5.55 ± 0.05 mg rutin g^{-1} dw in callus observed at extraction treatment T15. The maximum TFC in case of pummelo callus (2.67 ± 0.03 mg rutin g^{-1} dw) was observed for T6 (E:W ratio of 25 at 50 °C for 30 min) which was statistically at par with treatment T15 (2.6 ± 0.06 mg rutin g^{-1} dw). The TFC of three citrus

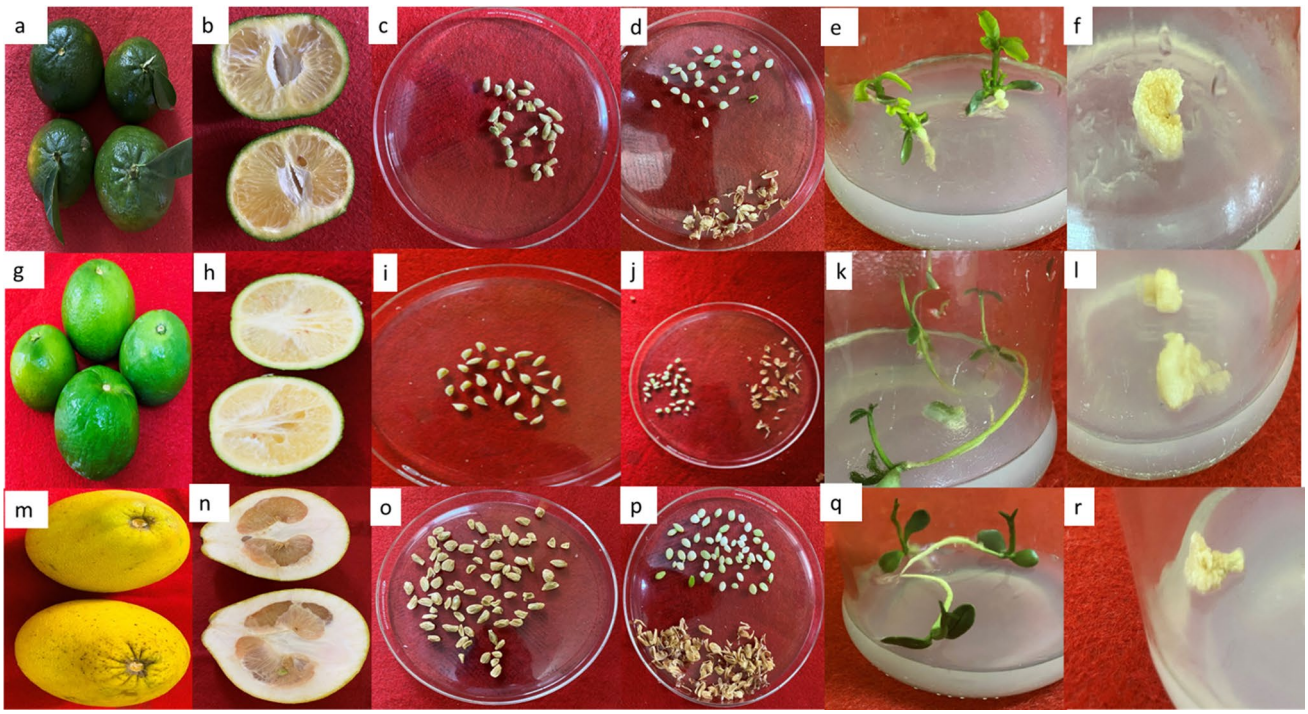

Fig. 1 Various stages of seed germination and callus induction in **a–f** Mandarin, **g–h** Sweet orange and **m–r** Pummelo. (**a**, **b**, **g**, **h**, **m**, **n**: Fruits; **c**, **d**, **i**, **j**, **o**, **p**: Seeds and removed testa; **e**, **k**, **q**: explants; **f**, **l**, **r**: Callus obtained)

Table 2 Effect of different extraction conditions on total phenol content in leaf and callus extracts of citrus species

Total phenol content (mg gallic acid g^{-1} dw)						
Treatment	Pummelo		Sweet orange		Mandarin	
	Leaves	Callus	Leaves	Callus	Leaves	Callus
T1	3.02 ± 0.03o	2.59 ± 0.13p	5.42 ± 0.01m	4.75 ± 0.05o	7.25 ± 0.04q	6.35 ± 0.06l
T2	3.18 ± 0.01mno	2.73 ± 0.04o	6.23 ± 0.04l	5.57 ± 0.06k	8.05 ± 0.05no	7.32 ± 0.03ij
T3	3.7 ± 0.15k	2.98 ± 0.13lmn	6.87 ± 0.02ij	6.01 ± 0.04i	8.77 ± 0.05ij	7.86 ± 0.06g
T4	4.75 ± 0.15g	3.13 ± 0.02ijkl	7.35 ± 0.04fg	5.82 ± 0.04j	8.15 ± 0.05n	7.25 ± 0.05j
T5	5.04 ± 0.03ef	3.56 ± 0.03d	7.96 ± 0.04e	6.35 ± 0.06h	9.2 ± 0.1f	7.86 ± 0.05g
T6	5.38 ± 0.07d	4.8 ± 0.04a	8.36 ± 0.03d	6.75 ± 0.03ef	9.85 ± 0.02c	8.54 ± 0.04d
T7	3.47 ± 0.04l	2.97 ± 0.05mn	6.21 ± 0.07l	5.22 ± 0.06lm	7.85 ± 0.03p	6.85 ± 0.05k
T8	3.96 ± 0.05ij	3.34 ± 0.03efgh	7.18 ± 0.07h	5.95 ± 0.03ij	8.35 ± 0.04m	7.58 ± 0.06h
T9	4.75 ± 0.19g	3.88 ± 0.04c	7.45 ± 0.04f	6.35 ± 0.07h	8.95 ± 0.04gh	8.11 ± 0.03f
T10	4.02 ± 0.17i	2.93 ± 0.04n	6.75 ± 0.04j	5.15 ± 0.05m	9.05 ± 0.07g	7.86 ± 0.05g
T11	4.33 ± 0.05h	3.1 ± 0.04jklm	7.25 ± 0.05gh	6.35 ± 0.04h	9.04 ± 0.03g	8.25 ± 0.05e
T12	4.88 ± 0.05fg	3.47 ± 0.03de	8.37 ± 0.01d	6.89 ± 0.07d	9.65 ± 0.05d	8.86 ± 0.06c
T13	5.25 ± 0.06d	3.56 ± 0.05d	8.45 ± 0.06d	6.83 ± 0.02de	8.85 ± 0.03hi	8.25 ± 0.05e
T14	5.89 ± 0.07b	3.79 ± 0.03c	9.28 ± 0.04b	7.25 ± 0.06c	9.38 ± 0.04e	8.89 ± 0.08c
T15	6.86 ± 0.04a	4.32 ± 0.07b	9.78 ± 0.03a	8.17 ± 0.04a	10.76 ± 0.03a	9.24 ± 0.04a
T16	4.75 ± 0.04g	3.27 ± 0.06fghi	7.24 ± 0.05gh	6.24 ± 0.04h	8.56 ± 0.02kl	8.06 ± 0.06f
T17	5.22 ± 0.08de	3.49 ± 0.04de	8.36 ± 0.04d	6.75 ± 0.04ef	9.25 ± 0.04ef	8.47 ± 0.07d
T18	5.59 ± 0.06c	3.91 ± 0.04c	8.78 ± 0.06c	7.38 ± 0.08b	9.79 ± 0.07c	9.04 ± 0.02b
T19	3.15 ± 0.03no	2.79 ± 0.06o	6.13 ± 0.04l	4.95 ± 0.05n	7.86 ± 0.06p	6.95 ± 0.05k
T20	3.29 ± 0.02lmn	3.04 ± 0.05klmn	6.98 ± 0.06i	5.85 ± 0.09j	8.65 ± 0.05jk	7.42 ± 0.03i
T21	3.68 ± 0.01k	3.22 ± 0.05hij	7.45 ± 0.05f	6.07 ± 0.05i	9.04 ± 0.04g	8.51 ± 0.05d
T22	3.38 ± 0.05lm	3.17 ± 0.06ijk	6.87 ± 0.03ij	5.86 ± 0.06j	8.47 ± 0.03l	7.75 ± 0.04g
T23	3.47 ± 0.02l	3.38 ± 0.05efg	7.25 ± 0.03gh	6.55 ± 0.05g	9.35 ± 0.06e	8.26 ± 0.06e
T24	3.78 ± 0.03jk	3.76 ± 0.06c	7.89 ± 0.04e	7.22 ± 0.03c	10.01 ± 0.06b	8.56 ± 0.06d
T25	3.24 ± 0.03mn	3.04 ± 0.03klmn	6.48 ± 0.07k	5.33 ± 0.02l	7.98 ± 0.06o	7.87 ± 0.07g
T26	3.36 ± 0.03lmn	3.26 ± 0.05ghi	7.43 ± 0.05f	6.05 ± 0.05i	8.75 ± 0.05ij	8.07 ± 0.04f
T27	3.47 ± 0.02l	3.42 ± 0.05def	7.86 ± 0.08e	6.68 ± 0.01f	9.25 ± 0.05ef	8.87 ± 0.06c

The values are mean of three replicates. Values after ± denote standard deviation. Dissimilar alphabets denote significant differences among treatments at $p < 0.05$ level of significance

species decreased when the callus formation was induced as the leaf tissues showed higher TFC as shown in Table 3.

The maximum RSA in the leaf tissue and callus cultures of three citrus species were also observed for T15 with E:W ratio of 50 at 50 °C for 30 min (Table 4) showing significant difference from other treatments at $p < 0.05$ level of significance. The RSA in case of pummelo was 42 ± 0.2% and 32.2 ± 0.8% in leaf tissue and callus respectively. In case of sweet orange, the RSA in leaf tissue and callus were 43.5 ± 1.3% and 40.5 ± 0.5% respectively. In case of mandarin, the RSA in leaf tissue and callus were 50.5 ± 0.9% and 47.8 ± 0.8% respectively. The leaf tissues exhibited higher RSA as compared to callus cultures in all the citrus species. The analysis of variance among different extraction conditions is shown in Table S1. The results showed that the treatments differed significantly from each other at $p < 0.001$ level of significance.

The results suggested that the maximum extraction of phenolic compounds was achieved from mandarin as compared to pummelo and sweet orange. Moreover, the leaf tissue exhibited higher phenols and flavonoids with higher antioxidant potential as compared to callus cultures. Similar results were reported by Yang et al. [30] who reported decrease in TPC and TFC of callus extracts as compared to root tissues in case of *Helicteres angustifolia L.* Similarly, Amid et al. [31] reported a decrease in TPC of callus cultures as compared to leaf tissue in methanolic and aqueous extracts in case of *Justicia gendarusa*. This suggested that the callus cultures could be used as an alternative source for the production of phenols and flavonoids by optimizing culture and extraction conditions to increase their production.

Table 3 Effect of different extraction conditions on total flavonoid content in leaf and callus extracts of citrus species

Total flavonoid content (mg rutin g^{-1} dw)						
Treatment	Pummelo		Sweet orange		Mandarin	
	Leaves	Callus	Leaves	Callus	Leaves	Callus
T1	1.68 ± 0.03q	1.44 ± 0.03mn	3.25 ± 0.04n	2.85 ± 0.03n	4.35 ± 0.04s	3.71 ± 0.03n
T2	1.72 ± 0.02pq	1.52 ± 0.01lmn	3.84 ± 0.02l	2.98 ± 0.01km	4.68 ± 0.06r	4.42 ± 0.02kl
T3	1.95 ± 0.02ijk	1.66 ± 0.03hijk	4.12 ± 0.03k	3.56 ± 0.05hi	5.28 ± 0.07klm	4.71 ± 0.04h
T4	2.16 ± 0.04ef	1.74 ± 0.03efghij	4.41 ± 0.05i	3.49 ± 0.03i	4.89 ± 0.02pq	4.35 ± 0.05l
T5	2.72 ± 0.03c	1.98 ± 0.04c	4.86 ± 0.04f	3.71 ± 0.05fgh	5.52 ± 0.03ghi	4.76 ± 0.05gh
T6	3.14 ± 0.02b	2.67 ± 0.03a	5.23 ± 0.02d	4.05 ± 0.05cd	5.91 ± 0.04c	5.24 ± 0.04cd
T7	1.93 ± 0.03ijkl	1.65 ± 0.04ijkl	3.58 ± 0.07m	2.97 ± 0.02km	4.71 ± 0.03r	4.15 ± 0.05m
T8	2.2 ± 0.03e	1.86 ± 0.03cdef	4.25 ± 0.04j	3.62 ± 0.02ghi	5.11 ± 0.07no	4.55 ± 0.04ij
T9	2.64 ± 0.03d	2.14 ± 0.03b	4.58 ± 0.05gh	3.83 ± 0.07ef	5.44 ± 0.15hij	4.86 ± 0.06fg
T10	1.82 ± 0.02mno	1.63 ± 0.04jkl	4.02 ± 0.06k	3.07 ± 0.04kl	4.93 ± 0.04p	4.71 ± 0.05h
T11	1.97 ± 0.04hij	1.72 ± 0.05fghij	4.47 ± 0.04hi	3.82 ± 0.02ef	5.42 ± 0.02ijk	4.95 ± 0.07f
T12	2.22 ± 0.04e	1.93 ± 0.04cd	5.14 ± 0.03d	4.34 ± 0.04b	5.79 ± 0.05cd	5.31 ± 0.02bc
T13	2.11 ± 0.04fg	1.98 ± 0.01c	5.37 ± 0.04c	4.1 ± 0.1c	5.31 ± 0.03jkl	4.95 ± 0.05f
T14	2.24 ± 0.03e	2.11 ± 0.06b	5.65 ± 0.04b	4.37 ± 0.03b	5.68 ± 0.07def	5.37 ± 0.04b
T15	3.25 ± 0.03a	2.6 ± 0.06a	5.94 ± 0.07a	4.92 ± 0.02a	6.56 ± 0.05a	5.55 ± 0.05a
T16	1.9 ± 0.03jklm	1.82 ± 0.06defg	4.64 ± 0.04g	3.74 ± 0.02fg	5.16 ± 0.05mno	4.36 ± 0.06l
T17	2.09 ± 0.03fg	1.93 ± 0.02cd	5.41 ± 0.04c	4.05 ± 0.05cd	5.64 ± 0.02efg	5.08 ± 0.07e
T18	2.24 ± 0.03e	2.17 ± 0.06b	5.68 ± 0.06b	4.42 ± 0.03b	5.78 ± 0.01cde	5.42 ± 0.03b
T19	1.76 ± 0.03op	1.55 ± 0.04klm	3.78 ± 0.03l	2.97 ± 0.04km	4.76 ± 0.04qr	4.18 ± 0.07m
T20	1.83 ± 0.01mno	1.69 ± 0.04ghijk	4.27 ± 0.04j	3.51 ± 0.06i	5.19 ± 0.06lmno	4.52 ± 0.05jk
T21	1.99 ± 0.03hi	1.79 ± 0.05defghi	4.55 ± 0.03gh	3.75 ± 0.22fg	5.42 ± 0.03ijk	5.16 ± 0.04de
T22	1.86 ± 0.02lmn	1.67 ± 0.02hijk	4.12 ± 0.07k	3.32 ± 0.04j	5.08 ± 0.06o	4.65 ± 0.05hi
T23	1.93 ± 0.02ijkl	1.78 ± 0.03efghi	4.38 ± 0.06i	3.93 ± 0.04de	5.65 ± 0.05efg	4.95 ± 0.01f
T24	2.1 ± 0.05fg	1.88 ± 0.02cde	4.99 ± 0.03e	4.33 ± 0.05b	6.09 ± 0.06b	5.16 ± 0.03de
T25	1.8 ± 0.04no	1.4 ± 0.17n	3.88 ± 0.04l	3.18 ± 0.03jk	4.78 ± 0.05qr	4.72 ± 0.02h
T26	1.87 ± 0.03klmn	1.72 ± 0.03	4.57 ± 0.02gh	3.63 ± 0.02ghi	5.25 ± 0.03lmn	4.85 ± 0.04fg
T27	2.04 ± 0.02gh	1.8 ± 0.04defgh	4.84 ± 0.03f	4.08 ± 0.08cd	5.57 ± 0.03fgh	5.34 ± 0.04bc

The values are mean of three replicates. Values after ± denote standard deviation. Dissimilar alphabets denote significant differences among treatments at $p < 0.05$ level of significance

Optimization of extraction conditions

Various methods are involved in the extraction of phenolic compounds and recovery of antioxidants from plants such as Soxhlet extraction, maceration, supercritical fluid extraction, subcritical water extraction, and ultrasound-assisted extraction [32]. The amount of phenolics extracted and their antioxidant potential is determined both by extraction method and the type of solvent used. The different phenolic compounds present in plant tissues may possess different chemical characteristics and thus are soluble in different chemicals [33]. For example, polyphenols are recovered using polar solvents which are aqueous mixtures containing alcohols such as ethanol and methanol, acetone, and ethyl acetate [32]. Ethanol is considered a good solvent for polyphenol extraction as it is safe for human consumption [34]. The E:W solvents are considered effective in extracting phenolic compounds than water, and the previous studies have reported that extracts prepared in ethanol exhibited a higher antioxidant activity than aqueous extracts [35].

The optimal E:W ratio was shown to be crucial in achieving better TPC and TFC yields from both leaves tissue and callus cultures. The E:W ratio of 50 yielded the maximum amount of phenolic compounds which was significantly higher than those at E:W ratio of 25 and 75 (Fig. 2a).The extraction of phenolic compounds from callus extracts significantly increased with increase in ultrasonic temperature to 50 °C, and the further increase in temperature to 70 °C significantly decreased the amount of phenolic compounds (Fig. 2b). In both callus cultures and leaf tissue extracts, the extraction of phenolic compounds increased significantly with the increase in extraction time (Fig. 2c) with the maximum extraction occurring at 30 min extraction time.

Table 4 Effect of different extraction conditions on radical scavenging activity in leaf and callus extracts of citrus species

Radical scavenging activity (%)						
Treatment	Pummelo		Sweet orange		Mandarin	
	Leaves	Callus	Leaves	Callus	Leaves	Callus
T1	12.2 ± 0.5m	10.53 ± 0.45i	18.8 ± 0.7n	15.4 ± 0.4m	24.5 ± 0.4o	20.5 ± 0.7l
T2	15.5 ± 0.5l	11.8 ± 0.9i	20.5 ± 1mn	17.4 ± 0.6l	25.9 ± 0.8no	22.8 ± 0.8jkl
T3	20.2 ± 0.17ij	15 ± 0.7h	24.6 ± 1.2ijk	20.8 ± 0.7jk	30.2 ± 1.1jkl	25.5 ± 0.8hij
T4	20.8 ± 0.3hi	18.5 ± 0.9fg	22.9 ± 0.96klm	20.4 ± 0.4jk	28.8 ± 0.8lmn	24.5 ± 0.5jk
T5	28.53 ± 0.25d	22.5 ± 0.8de	25.6 ± 0.5hij	22.5 ± 0.5hi	31.5 ± 0.9ijkl	27.8 ± 0.8ghi
T6	36.8 ± 0.35b	25 ± 1c	29.5 ± 1.47ef	26.5 ± 0.6e	35.5 ± 1.4fgh	31.5 ± 1.5e
T7	17.8 ± 0.2k	15 ± 0.8h	20.5 ± 0.36mn	17.4 ± 0.44l	27 ± 1mno	23.8 ± 0.9jk
T8	24.5 ± 0.2ef	22 ± 1.04de	23.5 ± 0.66jkl	20.5 ± 0.3jk	29.5 ± 1.1klm	25 ± 1.4ij
T9	29 ± 0.3d	25.07 ± 1c	25.6 ± 0.7hij	24.5 ± 0.6fg	32.5 ± 0.5hijk	28.5 ± 1.1fgh
T10	18.5 ± 0.87jk	16 ± 1.5h	23.4 ± 1.1jkl	20.4 ± 0.4jk	29.5 ± 0.8klm	25.6 ± 0.6hij
T11	22.5 ± 0.2fgh	18.5 ± 0.6fg	26.8 ± 0.9ghi	23.5 ± 0.7gh	32.5 ± 0.7hijk	28.2 ± 1.2fgh
T12	28.4 ± 0.17d	23.5 ± 0.5cd	31.2 ± 0.53e	28.4 ± 0.4d	37.8 ± 0.8def	34.5 ± 0.5cd
T13	23.2 ± 0.2fg	20.5 ± 0.7ef	27.8 ± 1.3fgh	24.5 ± 0.3fg	33.5 ± 0.5hi	30.5 ± 1.4efg
T14	32.6 ± 0.1c	28.9 ± 0.8b	37.6 ± 1bc	34.8 ± 0.8b	42.8 ± 1.5bc	38.5 ± 0.9b
T15	42 ± 0.2a	32.2 ± 0.8a	43.5 ± 1.3a	40.5 ± 0.5a	50.5 ± 0.9a	47.8 ± 0.8a
T16	20.5 ± 0.4hij	16.9 ± 1.25gh	25.8 ± 0.7hij	22.6 ± 0.6hi	31.5 ± 0.5ijkl	27.8 ± 1.1ghi
T17	25.8 ± 0.4e	22.5 ± 0.5de	29.8 ± 1.18ef	25.8 ± 0.6ef	36.5 ± 0.9efg	32.4 ± 1.4de
T18	34 ± 1.73c	28 ± 1b	36.6 ± 1.2bcd	32.5 ± 0.4c	42.5 ± 0.8bc	38.7 ± 1.5b
T19	15 ± 0.7l	12.5 ± 0.7i	21.8 ± 0.4lm	19.5 ± 0.8k	26.5 ± 0.7no	22.1 ± 1.1kl
T20	18 ± 1.04k	15 ± 0.5h	24.5 ± 0.96ijk	21.5 ± 0.5bij	30.2 ± 0.7jkl	27.8 ± 1.4ghi
T21	25.5 ± 0.7e	20.5 ± 0.8ef	28.5 ± 0.17fg	25.8 ± 0.6ef	34.2 ± 1.1ghi	31 ± 0.8ef
T22	21.8 ± 1.32ghi	15.5 ± 0.3h	27.5 ± 0.9fgh	24.5 ± 0.3fg	33.2 ± 1.3hij	28.17 ± 1.04fgh
T23	29.23 ± 0.85d	20.5 ± 0.5ef	34.5 ± 0.5d	31.7 ± 0.4c	40.5 ± 2.5cd	33.2 ± 1.3cde
T24	34 ± 0.8c	25.37 ± 1.35c	38.5 ± 0.87b	35.6 ± 0.2	44.8 ± 1.2b	38.5 ± 1.1b
T25	24 ± 1.1ef	20.2 ± 0.6ef	25.5 ± 0.35hij	22.5 ± 0.5hi	31.5 ± 1.5ijkl	27.5 ± 0.7ghi
T26	29 ± 0.9d	25.5 ± 0.6c	31.07 ± 1.5e	28.4 ± 0.26d	38.5 ± 1.1de	33.4 ± 1.22cde
T27	37.8 ± 2.11b	30 ± 1b	35.5 ± 0.5cd	31.8 ± 0.3c	40.5 ± 1.6cd	35.5 ± 0.8c

The values are mean of three replicates. Values after ± denote standard deviation. Dissimilar alphabets denote significant differences among treatments at $p < 0.05$ level of significance

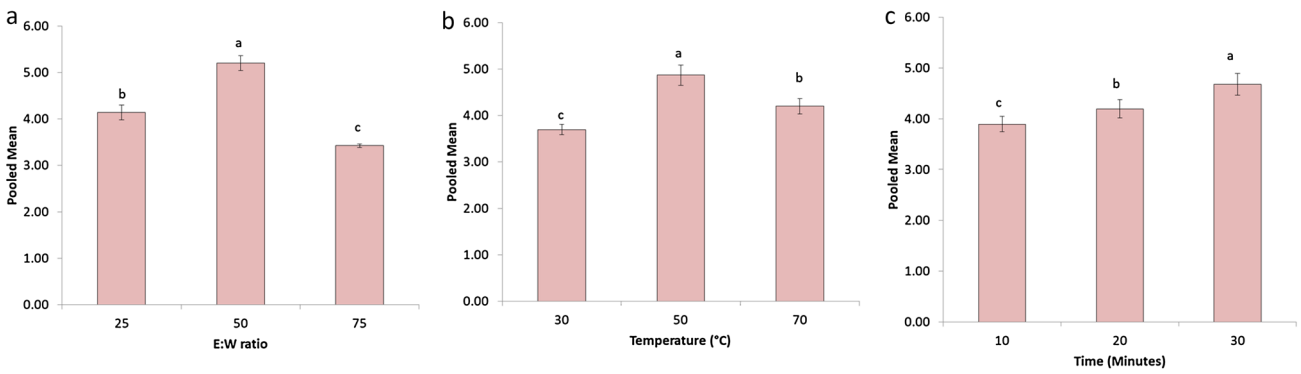

Fig. 2 Effect of **a** E:W ratio **b** temperature and **c** time on pooled mean of TPC, TFC and RSA in leaf tissue and callus of three citrus species. The error bars represent standard error and dissimilar alphabets represent significantly different data at $p < 0.05$ level of significance

The mean effects of interaction between various extraction parameters are provided as supplementary data (Table S2–S5). The interaction of E:W ratio with temperature revealed that the maximum extraction could be achieved with E:W ratio of 50 at 50 °C (Table S2). The interaction between E:W ratio and time showed that the maximum extraction was achieved when extraction was carried out with E:W ratio of 50 for 30 min (Table S3). Similarly, interaction between temperature and time revealed that carrying out the extraction at 50 °C for 30 min would result in the maximum extraction (Table S4). The triple interaction among the three parameters (E:W ratio, temperature and time) showed that E:W ratio of 50 at 50 °C for 30 min would yield in the maximum extraction of phenolic compounds (Table S5). The analysis of variance showed that the interactions were statistically significant at $p < 0.001$ level of significance (Table S6).

Similar results were reported by Jimenez-Monero et al. [36] in which phenolic compounds from grape stem extracts were extracted and it was observed that the extraction using 50% ethanol resulted in the maximum amount of TPC which decreased when 25% and 75% ethanol solution was used. Also, the extraction was reported to be higher at 40 °C as compared to 25 °C. Bonoli et al. [37] obtained higher TPC from barley flour by using an extraction mixture of ethanol and acetone. Turkmen et al. [33] obtained extracts with the highest antioxidant potential by using 50% aqueous ethanol in the case of mate tea.

The ultrasound-assisted extraction was employed in the present study. The cell wall lysis in ultrasound water baths is carried out by shear forces created by cavitation which break the cell walls and sonication helps in diffusion of the cell contents into the extraction solution [38]. Furthermore, this method can reduce the extraction time and amount of solvent required while providing the maximum recovery of phenolic compounds from plants [39]. Jerman et al. [40] showed that extraction via ultrasonication enhances the yield of phenolic compounds by up to 80% as conventional solvent extraction in case of olive. The amount of extracted phenols and their RSA was observed to be maximum at 50 °C (Fig. 2b) which decreased at 70 °C. The decrease in phenolic extraction from callus and leaves at higher temperatures might be due to phenolic breakdown into other chemical components at high temperature.

Correlation between TPC or TFC of leaf tissue and callus and their RSA

Correlation analysis was carried out to study whether the phenols or flavonoids contributed to the antioxidant capacity of the leaf tissue and callus in three citrus species. Pearson's correlation coefficients were analyzed in three citrus species for leaf tissues and callus cultures (Table S7). In case of pummelo, the highest degree of correlation was observed between TPC and RSA of callus (r = 0.80431). In case of sweet orange both TPC and TFC of callus contributed towards RSA of callus with r = 0.88619 and r = 0.88738 respectively. In case of mandarin, the highest degree of correlation was observed with TPC and TFC of leaves with RSA of leaves with r = 0.89359 and r = 0.89928 respectively (Table S6). The scatter plots of the correlation analysis are shown in Fig. 3. Zhang et al. [41] performed correlation analysis between TPC and TFC of different fruit tissue in mandarin and found that the TPC had higher degree of correlation with DPPH as compared to TFC. Flavonoids are reported as the main phenolic compounds in citrus [41]. This might be the reason for high correlation coefficients of TFC with RSA in case of sweet orange and mandarin [9–12]. In case of pummelo leaf tissue, both TPC and TFC showed lower correlation coefficients (r = 0.60547 and r = 0.72787 respectively) as compared to callus cultures (Table S7). This could be due to the fact that the specific type of phenolic compounds contributing towards antioxidant potential could be higher in callus cultures as compared to control. Similar results were observed in case of sweet orange where the correlation coefficients were higher in callus cultures as compared to leaf tissue (Table S7).

Principal component analysis

The principal component analysis was carried out using SAS studio to identify the parameters which were the most affected by the different extraction conditions. The component patterns are shown in Fig. 4. The first two principal components could explain about 91.67% of the variance with all the parameters strongly influencing component 1. The variables lying across X-axis i.e. % RSA of leaf tissue and callus culture in pummelo, and TPC and TFC of leaf tissue in mandarin and sweet orange and callus culture in sweet orange, were the most affected(Fig. 4).The % RSA in case of pummelo leaves and callus were more strongly affected by the extraction conditions as compared to the % RSA in case of sweet orange and mandarin leaves and callus. In case of sweet orange leaves,the TPC and TFC were strongly correlated with each other while their correlation with % RSA was not observed. The TPC of the pummelo leaves was the least affected parameter. The % RSA of sweet orange leaves and callus showed strong correlation with % RSA of mandarin leaves. Similarly, the TPC and TFC of mandarin callus were highly correlated with each other.

Conclusion

The present study was conducted to investigate the impact of extraction solvent, ultrasonic temperature and time on TPC, TFC and RSA of leaf tissue and callus extracts of

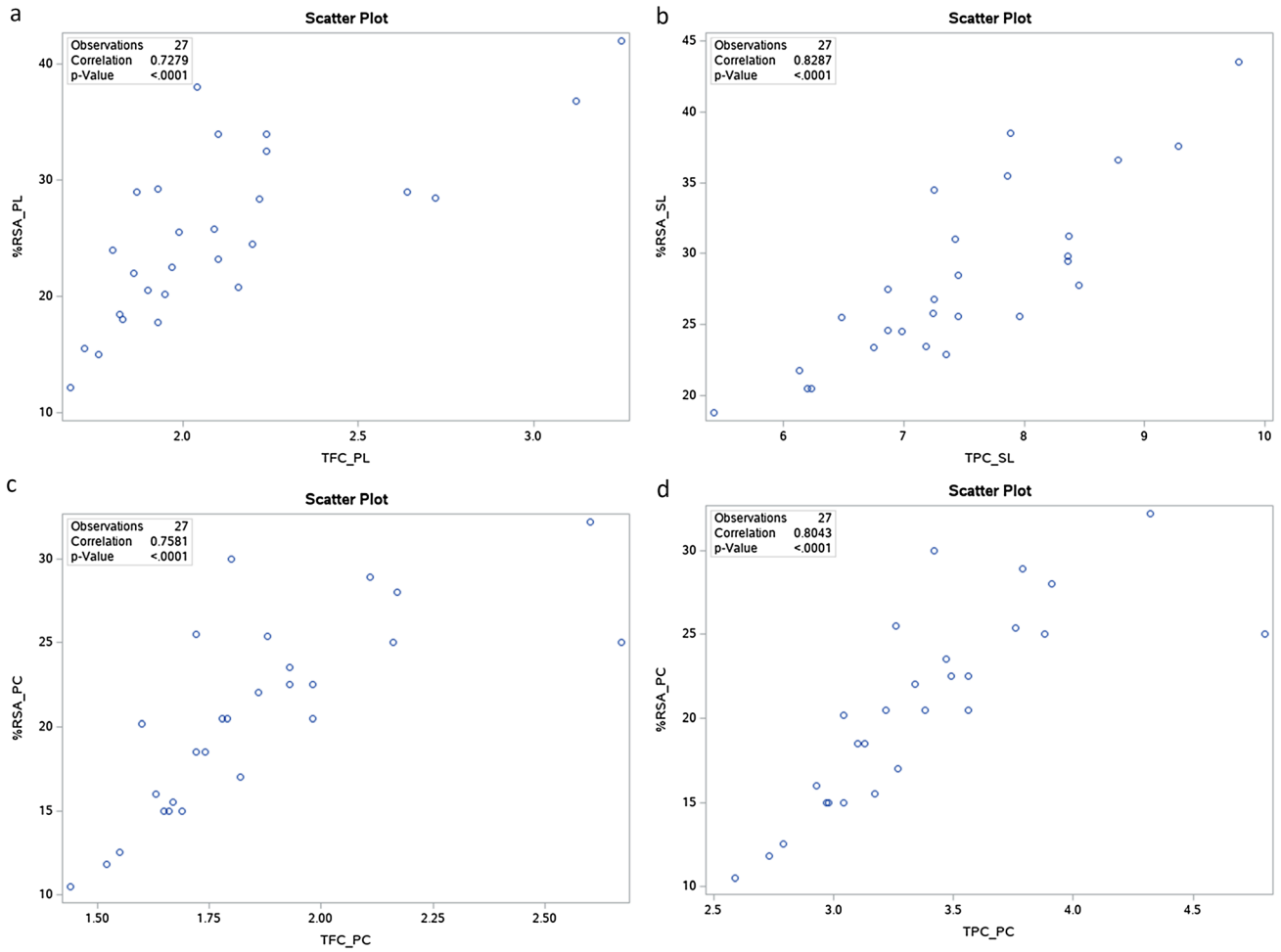

Fig. 3 Scatter plots for correlation analysis between **a** TFC of leaf tissue (TFC_PL) and %RSA of leaf tissue (%RSA_PL) in pummelo. **b** TPC of leaf tissue (PTC_SL) and %RSA of leaf tissue (%RSA_SL) in sweet orange. **c** TFC of callus (TFC_PC) and %RSA (%RSA_PC) of callus in pummelo. **d** TPC of callus (TPC_PC) and %RSA of callus (%RSA_PC) in pummelo

three citrus species: pummelo, sweet orange and mandarin. It was found that leaf tissues exhibited higher amount of TPC, TFC and RSA as compared to callus cultures. The amount of phenolic compounds from callus cultures could be increased by further optimization of extraction conditions and extraction solvents. The use of callus cultures would help in extraction of secondary metabolites all around the year irrespective of the seasonal variations, microbial pests, diseases and geographical constraints which affect the availability of plant tissues. Calli, mass of cells carries the DNA similar to the mother plant and therefore, has the efficiency for the production of bioactive compounds similar to the mother plant. Hence, calli can be used for the isolation of phytochemicals of interest without damaging the entire plant. These callus cultures can be changed into cell suspension cultures for bio-fermentors to produce secondary metabolites of interest. Thus, the leaf tissues as well as callus cultures of citrus plants could be an important source of bioactive compounds which could be used in various bio-active products.

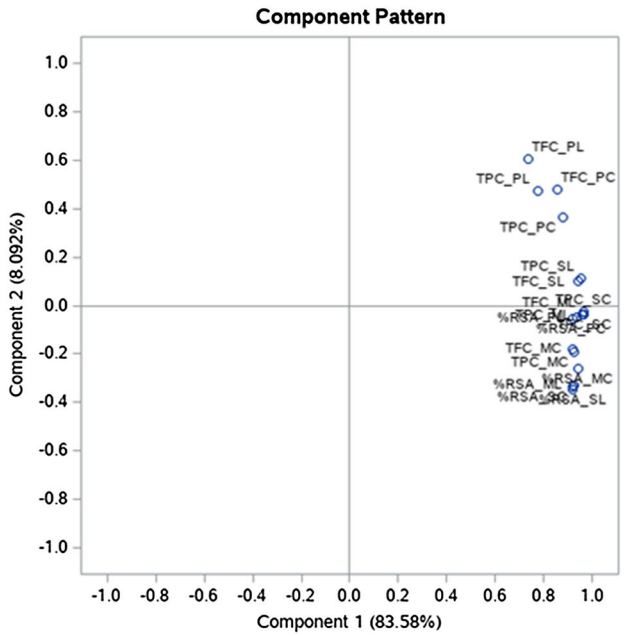

Fig. 4 Principal component analysis of various parameters (TPC_PL: TPC of leaf tissue in pummelo; TPC_PC: TPC of callus culture in pummelo; TFC_PL: TFC of leaf tissue in pummelo; TFC_PC: TFC of callus culture in pummelo; %RSA_PL: %RSA of leaf tissue in pummelo; %RSA_PC: %RSA of callus culture in pummelo; TPC_ML: TPC of leaf tissue in mandarin; TPC_MC: TPC of callus culture in mandarin; TFC_ML: TFC of leaf tissue in mandarin; TFC_MC: TFC of callus culture in mandarin; %RSA_ML: %RSA of leaf tissue in mandarin; %RSA_MC: %RSA of callus culture in mandarin; TPC_SL: TPC of leaf tissue in sweet orange; TPC_SC: TPC of callus culture in sweet orange; TFC_SL: TFC of leaf tissue in sweet orange; TFC_SC: TFC of callus culture in sweet orange; %RSA_SL: %RSA of leaf tissue in sweet orange; %RSA_SC: %RSA of callus culture in sweet orange) (Color figure online)

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11694-022-01695-6.

Acknowledgements The authors thank Director, School of Agricultural Biotechnology, College of Agriculture for providing necessary funding and infrastructure to carry out the research work.

Data availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

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