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Analytical Methods

Ultrasonic-assisted extraction and UHPLC determination of ascorbic acid, polyphenols, and half-maximum effective concentration in *Citrus medica* and *Ziziphus spina-christi* fruits using multivariate experimental design

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ABSTRACT

This study aimed to determine the concentrations of ascorbic acid and polyphenols in fruits and peels of *Citrus medica* and *Ziziphus spina-christi* grown in Ethiopia. Conditions of ultrasound-assisted extraction (UAE) and ultra-high-performance liquid chromatography coupled with a diode array detector (HPLC-DAD) were optimized, using a multivariate experimental design. The optimum conditions of UAE were 15 min extraction time at 35 ℃, with 75 % aqueous methanol as solvent, and a fruit powder-to-solvent ratio (m/v) of 1:15. Among the different drying conditions investigated, freeze-drying was found to be appropriate for analyzing ascorbic acid, polyphenols, and antioxidant potential. The overall ranges, across the fruits and peels, of ascorbic acid, total polyphenols, and antioxidant potentials (EC50) obtained were 8.7 ± 1.4–91.2 ± 2.6 mg/100 g, 253.0 ± 6.3–764.1 ± 25.8 mg GAE/100 g and 2.4 ± 0.1–26.1 ± 2.9 mg/mL, respectively. This indicates that the fruits and peels of the studied plants are advantageous as sources of ascorbic acid and polyphenols.

Introduction

In vitro and *in vivo* experimental studies show that consuming large amounts of fruits and vegetables is associated with a lower prevalence of risk factors for cardiovascular diseases, hypertension, obesity, type 2 Diabetes mellitus, and stroke (Rossi et al., 2018). *Citrus* fruits are one of the main sources of ascorbic acid and other antioxidant compounds, such as phenolic compounds. Moreover, studies indicate that the consumption of mainly *Citrus* fruits could prevent cancer and is associated with reduced overall cancer incidence (Bae & Kim, 2016). Consuming fruits such as those from *Citrus medica* (Herrera-Pool et al., 2021) and *Ziziphus spina-christi* (Taghipour et al., 2020) plants can treat several diseases, including Covid-19, because of the availability of bio-nutrients such as ascorbic acid. The *C. medica* and *Z. spina-christi* plants are commonly found throughout various regions of Ethiopia (Du et al., 2013). These plants produce abundant fruits, which are readily available and can be harvested extensively across the country. Despite the ample availability of these the fruits are underutilized. Their utilization and consumption vary from region to region within Ethiopia. These differences persist due to the absence of thorough research and a lack of awareness regarding the nutritional value of these plants within society. By addressing these gaps in knowledge and understanding, our research aims to shed light on the potential benefits and value of these plants as a valuable dietary resource. Through enhanced awareness and appreciation of their nutritional significance, we hope to encourage more widespread utilization of these plants across various regions of the

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country. As tropical fruits provide hidden sources of value-added products for society (Cerdá-Bernad et al., 2023), they are neglected despite their tremendous potential. In Ethiopia, these fruits have not yet been explored concerning their bio-nutrient compositions.

Ascorbic acid (vitamin C), polyphenols, and antioxidant potentials of fruits are selected due to their health benefits and importance in human nutrition (Oyarzún et al., 2020). Ascorbic acid is a naturally occurring, easily oxidized (González et al., 2019) strong antioxidant compound found in most plant species and their by-products, particularly grapes (Matei et al., 2013) and *Citrus* fruits (Chhikara et al., 2018). Ascorbic acid is a water-soluble vitamin that exists in a mono-protonated form in acidic conditions (Odriozola-Serrano et al., 2007). Out of this physiological condition, ascorbic acid decomposes into dehydroascorbic, isodehydroascorbic, and dehydro-isoascorbic acids (Boonpangrak et al., 2016). The content of ascorbic acid gradually decreases because of changes in pH (acidic or alkaline physiological conditions), storage temperature, and preservation methods (Odriozola-Serrano et al., 2007). Therefore, one of the common methods to preserve ascorbic acid and other bio-nutrients for a longer period is drying. Different researchers have attempted to investigate the effect of drying methods on the retention of bioactive components such as ascorbic acid in perishable foodstuffs like fruits and vegetables (Oyarzún et al., 2020). For instance, classical drying techniques like sun-drying (SD), room-drying (RD), and oven-drying, as well as modern drying methods such as vacuum freeze-drying (VFD), microwave-drying, and infrared-drying have been used for the preservation of ascorbic acid and other bioactive components of foods (Oyarzún et al., 2020). The reasons for choosing sun-drying, room-drying, and vacuum freeze-drying were by considering their ease of accessibility in different analytical laboratories. In addition, sun-drying and room-drying are commonly used to dry the studied fruits by the local community in Ethiopia.

Titration (Gorinstein et al., 2001), electrochemical (O'Connell et al., 2001), enzymatic (Shekhovtsova et al., 2006), spectrophotometric (Desai, 2019), capillary electrophoresis (Costa et al., 2019), and chromatographic separation methods (Orsavová et al., 2019) have all been used to determine ascorbic acid and other bio-nutrients in food matrices (Muhammad et al., 2018). More recently, automated voltammetry using 24-well microtiter plates, and "on–off-on" fluorescent nano-probes (Gong et al., 2017) have been used for the detection of ascorbic acid in different sample food matrices. Additionally, palladium nanoparticles supported on graphene oxide (Gong et al., 2017), flow–injection chemiluminescence (Ma et al., 2002), and amperometric L-ascorbic acid sensors (O'Connell et al., 2001) detection have been used for the determinations of ascorbic acid. However, the HPLC method of analysis is better than those of the aforementioned sensor-based detection of ascorbic acid because of interference in the latter methods if appropriate separation conditions for HPLC are chosen. There have also been few studies on ascorbic acid extraction and analysis using *meta*-phosphoric acid and buffer solutions made from mono- and di-basic phosphate salts (Odriozola-Serrano et al., 2007). Furthermore, past studies that focused on technique development, optimization, and validation for measuring ascorbic acid in food matrices using aqueous acetic acid are lacking. Therefore, a reliable method for the determination of ascorbic acid in fruits and vegetables needs to be developed and optimized.

The antioxidants and phenolic components of fruits and vegetables have been determined utilizing ultrasound-assisted extraction (UAE) and the combined response surface methodology (RSM). Because of its extraction effectiveness and reduced extraction time for polar, non-polar, and thermolabile bioactive components, UAE optimization using RSM is one of the most frequent procedures for bioactive components of foods and food products (Tabaraki et al., 2012). The use of RSM in experimental design is highly valuable for the development and optimization of analytical methods, including both extraction and high-performance liquid chromatography (HPLC) techniques (Almusallam et al., 2021). RSM allows for the simultaneous examination of multiple factors in a single study, making it a powerful mathematical and statistical tool for analyzing and optimizing independent variables that impact the outcomes of experiments. RSM not only provides insights into the individual effects of independent variables but also allows for the investigation of their interactions. This approach has been successfully applied to optimize various extraction conditions, such as those related to ascorbic acids, polyphenolic compounds, and other relevant factors in fruit extraction processes (Belwal et al., 2016). By considering and modeling the interaction effects, RSM enables researchers to gain a comprehensive understanding of the factors influencing the desired responses. Among the different types of models used in RSM, the FC-CCD model is particularly advantageous for optimizing extraction conditions. This model offers enhanced precision and accurate prediction capabilities, enabling researchers to fine-tune and improve the efficiency of the extraction process. The RSM has been applied mostly for the optimization of the experimental conditions for maximizing the recovery of total phenolic contents (TPC), and total flavonoid contents (TFC), and scavenging activities against 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Almusallam et al., 2021; Belwal et al., 2016). There are limited reported studies on the minimization effects of independent variables on effective concentration (EC50) values and the maximization effects of independent factors on ascorbic acid in food matrices using RSM.

Different assays can be used to measure the antioxidant activity of foods. It is commonly recommended to use at least two different assays to characterize the antioxidant capacity of a food sample (Sadowska-Bartosz & Bartosz, 2022). Nowadays, the antioxidant potentials (in terms of EC50), TPC, and TFC of fruits are the primary concerns of researchers in foods and food products. The DPPH,[2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)]-diammonium salt (ABTS), and ferric reducing antioxidant power (FRAP) free radical scavenging assays are the most common and widely used stable analytical methods to evaluate the antioxidant potential of foods (Belayneh et al., 2022). The estimations of the half maximum EC50 values have been studied using different mathematical and statistical models such as the dose–response, logistic, sigmoidal, asymmetric 5 parameters, GraphPad Prism, BLeSq, Origin, SigmaPlot, BioDataFit, IBM SPSS, and DPS (Chen et al., 2013; Sridhar & Linton, 2019). To the best of our knowledge, there are limited studies on the prediction of EC50 values of the selected fruits using a multi-concentration level GraphPad Prism statistical tool under different drying conditions.

As a result, the purpose of this study was to design and validate UAE and UHPLC-DAD techniques for evaluating ascorbic acid, polyphenols, and antioxidant activity of the fruits and peels of *C. medica* and *Z. spina-christi* using multivariate experimental design.

Hypothesis: The hypothesis suggests that wild edible fruits play a crucial role as rich sources of both ascorbic acid and antioxidants, contributing positively to human health. The composition of these fruits may be influenced by drying conditions and other independent variables, and optimizing these factors could potentially affect the fruit's nutritional profile.

Materials and methods

Samples

Fruit samples of *C. medica* and *Z. spina-christi* were gathered from Debre Sina located at 39°37′59.99″E and 12°9′0″N (North Shewa Zone) and Raya-Kobo at 39°45′45.64″E and 9°50′59.66″N (North Wollo Zone), Ethiopia. Mr. Abiyou Enyew of the Department of Biology, University of Gondar, Ethiopia, identified the botanical specimens of *Z. spina-christi* and *C. medica*, which were held at the Department's mini-herbarium under the voucher numbers 001/TBA/2020 and 004/TBA/2020, respectively.

The samples were placed in clean polyethylene plastic bags and delivered in an ice box to the National Institute of Food Technology, Entrepreneurship and Management, Haryana, India.

Sample pretreatment

The fruits were washed with distilled water, and the edible parts were isolated from the peels and seeds. The edible sections of the fruits and fruit peels of *C. medica* were then chopped into small pieces and divided into three groups for drying. The first portion was sun-dried (SD) in the range of 25.8 to 38 ℃, recorded between 9:00 a.m. to 3:30p.m. The second portion was dried and left opens in the air in the laboratory room (RD) within the temperature range of 19.9 to 23.7 ℃, recorded between 9:00 a.m. to 3:30p.m. The third portion was dried using a vacuum freeze-drier (VFD). Initially, the chopped fruits were put in thin layers on VFD trays and frozen at −40 ℃ in a deep freezer for 6 h. The initial temperatures of the VFD probes were set to −40 ℃ for 14 h, −20 ℃ for 20 h, and 0 ℃ for 14 h. The vacuum pressure and condenser temperature were both set at 1 Pa and −50 ℃.

Vacuum freeze-dried *C. medica* fruit samples were assigned as CMFfd, room-dried *C. medica* fruit as CMFrd, sun-dried *C. medica* fruit as CMFsd, freeze-dried *C. medica* peel as CMPfd, room-dried *C. medica* peel as CMPrd, sun-dried *C. medica* peel as CMPsd, freeze-dried *Z. spina-christi* fruit as SCFfd, room-dried *Z. spina-christi* fruit as SCFrd, and sun-dried *Z. spina-christi* fruit as SCFsd.

The dried fruit and peel samples were crushed to a fine powder (250 m sieve size) with a stainless steel grinder (700 g Electric Grains, China) and stored in a refrigerator at 4 ℃ until extraction and analysis.

Optimization of ultrasound-assisted extraction

Optimization using response surface methodology

Response surface methodology (RSM) was employed in five-level experimental designs based on face-centered central composite design (FC-CCD) with four independent parameters (time, temperature, methanol concentration, and fruit powder-to-solvent ratio) (Asfaw et al. 2023). These variables were utilized to optimize the ultrasound-assisted extraction (UAE) conditions (Ultrasonic cell crusher and intelligent ultrasonic processor, SJIA-950 W, China) for phenolic component extraction from fruits and peel samples. FC-CCD was used to investigate the effects of time (A) (5–25 min), temperature (B) (20–45 ℃), and percent methanol in water (Purelab flex 4 Elga, USA) (C) (45–100 %) on the extraction and analysis of total phenolics content (TPC), total flavonoid content (TFC), and antioxidant activity (EC50).

Similarly, using 1 % aqueous acetic acid as the solvent, the effects of time (A) (5–25 min), temperature (B) (10–30 ℃), and sample mass-to-solvent ratio (C) (5–25) on ascorbic acid determination were examined. After gross estimations of the responses, CMFrd, CMPfd, and SCFfd were employed for the optimization processes. Furthermore, the DPPH assay was utilized to optimize the EC50 values. Table 1 shows the actual and coded levels of components, as well as the quadratic regression equation, which incorporates all interactions. According to Equation (1), all interactions were considered to determine the predicted response:

$$Y = \beta_o + \sum_{i=1}^{n}\beta_i A + \sum_{j=1}^{n}\beta_j B + \sum_{k=1}^{n}\beta_k C + \sum_{l=1}^{n}\beta_l D + \sum_{ii=1}^{n}\beta_{ii}A^2 + \sum_{jj=1}^{n}\beta_{jj}B^2 + \sum_{kk=1}^{n}\beta_{kk}C^2 + \sum_{ll=1}^{n}\beta_{ll}D^2 + \sum_{i\neq j=1}^{n}\beta_{ij}AB + \sum_{i\neq k=1}^{n}\beta_{ik}AC + \sum_{i\neq l=1}^{n}\beta_{il}AD + \sum_{j\neq k=1}^{n}\beta_{jk}BC + \sum_{j\neq l=1}^{n}\beta_{jl}BD + \sum_{k\neq l=1}^{n}\beta_{kl}CD + \varepsilon$$

where *Y* represents the response (TPC, TFC, EC50, and ascorbic acid); *A, B, C, and D* represent the independent variables; β_o represents the intercept; β_i, β_j,β_k, and β_l represent linear model term coefficients, β_{ii}, β_{jj},β_{kk}, and β_{ll} represent quadratic model term coefficients, β_{ij}, β_{ik}, βi_l, β_{jk}, βj_l, and β_{kl} represent model term interaction coefficients; n represents the number of variables (n = 4 for TPC and EC50, n = 3 for ascorbic acid); and ε represents the random error components that are normal and independently distributed with mean zero and constant variance.

The linear, quadratic, interaction effects, fitness, the level of significance (F-values at $p < 0.05$) tests, the lack of fit, the coefficient of determination (R^2), and the precision of the quadratic model were determined using analysis of variances (ANOVA). Further, validation experiments were carried out under optimized process conditions, and the percentage of relative error between predicted and experimental values of responses was calculated. To determine the validity of the model, the experimental values were compared with the predicted values based on the relative error or the coefficients of variations (CV %).

Three factorial points (-1, 0, +1) were employed in the FC-CCD model. The predicted outcomes for the updated models were used to generate response surface graphical representations at the 5 % confidence level. Experiments with UAE were carried out using the improved parameters to verify the mathematical model's prediction for all dependent variables (Table 1). The FC-CCD for TPC and EC50 had 30 trial runs (Supplementary files Table S2) with 6 center points and 24 non-center points. Furthermore, the FC-CCD for ascorbic acid included 20 experimental runs (Supplementary files Table S1), with 6 center points and 14 non-center points. Supplementary files (Tables S1 and S2) provide the experimental runs for each analysis (TPC, EC50, and ascorbic acid).

The FC-CCD model has five levels, each with two variables coded to be at ± 1 for the factorial points, (0,0) for the center point, and two axial points (±α) (Table 1). For TPC/EC50 and ascorbic acid, the distance between the axial and center points was ± 2 and ± 1.682, respectively. The distance between the axial and center points was determined as $\alpha = (2^n)^{1/4}$, where n is the number of variables. The codes were calculated as a function of each factor's range of interest. Experiments were carried out in a single block, with the order of runs inside the block randomized. The selection of optimum conditions was based on the high contents of ascorbic acid, TPC, and antioxidant activities based on the desirability function.

The content of ascorbic acid was determined using UHPLC-DAD (UltiMate 3000 Dionex, Thermo Scientific, Germany), while the antioxidant potentials (EC50), TPC, and TFC were determined using UV–VIS spectrophotometer (SHIMADZU, UV-2600, USA, and Jasco – V-770, Japan).

Ultrasound-Assisted extraction

After optimization, about 1 g of each of the dried samples was mixed with 15 mL of methanol in water (75/25, v/v). The extraction conditions for UAE were pulse on was 4 s, pulse off was 2 s, probe type was @6, and power was 50 % of 950 W. The sample extracts were centrifuged (Funke Gerber, Super Vario-N rotor with heating module, USA) at 3500 x g for

Table 1
Experimental variable ranges with coded levels using Face-centered central composite design.

Independent variables	Levels of coded variables for TPC and EC50				
	−α	Low	Center	High	+α
	−2	−1	0	+1	+2
Time (A), min	7.5	5	17.5	30	42.5
Temperature (B), ℃	7.5	25	35	45	57.5
Methanol Conc. (C), %	25	50	75	100	125
Fruit powder-to-solvent Ratio (D),	5	5	15	25	35
Independent variables	Levels of coded variables for ascorbic acid				
	−α	Low	Center	High	+α
	−1.682	−1	0	+1	+1.682
Time (A), min	5	5	12.5	20	20
Temperature (B), ℃	10	10	20	30	30
Fruit powder-to-solvent Ratio (C), Ratio	5	5	15	25	25

*Methanol Conc. is the percentage of methanol in water. Point of prediction tool; α value was 0.05, the Tolerance proportion was 0.99 and the lower or upper-value interval was two-sided.

15 min at room temperature. Then, a 75/25 methanol and water mixture was used to adjust the volume to 50 mL. Finally, the sample extracts were used to evaluate the TPC, TFC, DPPH, ABTS, FRAP activities, and EC50 values of the fruits and the peel.

Soxhlet extraction

The fruit and peel samples were also extracted using Soxhlet in accordance with the procedure described by Asfaw et al. (2023), for the purpose of comparison with the UAE. In brief, a 150 mL sample of methanol was used in a soxhlet extractor to extract a 10 g sample of dried fruit. A soxhlet extractor (Bucher, v16, Germany) was used to do the extraction at 50 ℃ for 4 h. The extracts were filtered using Whatman filter paper (No. 1) after being cooled to room temperature (23.7 ± 3 ℃). The volume was then changed to 50 mL and utilized to assess the antioxidant and phenolic activity of the fruit samples.

Extraction and determination of ascorbic acid using UHPLC-DAD

Ascorbic acid is the major vitamin constituent of citrus fruits. One of the studied fruits is a citrus fruit (*C. medica*), and hence its ascorbic acid content was measure and compared with the other fruits.

Extraction of ascorbic acid

In this study, an attempt was made to optimize conditions and develop an efficient extraction method for ascorbic acid using the UAE technique following the previously reported method by Asfaw et al. (2023). Various methods have been developed for the extraction and analysis of ascorbic acid in fruits and vegetables using phosphate buffer at pH between 2.5 and 3.5 as both extraction solvent and liquid chromatography mobile phase (Orsavová et al., 2019). Similarly, ascorbic acid has been extracted from carrots, beet, and cherry tomatoes using *meta*-phosphoric acid as solvent and liquid chromatography mobile phase (Chebrolu et al., 2012). In this study, preliminary trails using these methods, however, provided severe chromatographic base line drift as well as shoulder peaks on the chromatogram of ascorbic acid. Hence, from the different trials, 1 % aqueous acetic acid was found to be suitable for the extraction of ascorbic acid from the plant samples. As a result, 1 g of sample was mixed with 15 mL of 1 % aqueous acetic acid, and the mixture was extracted with UAE for 10 min at 25 ℃. An ice bath was utilized to regulate the extraction temperature. The extracted solution was centrifuged at 3500 × g for 15 min at room temperature. The supernatant was transferred to a 50 mL flask and adjusted to the correct concentration using 1 % aqueous acetic acid. The solution was then prepared for UHPLC-DAD analysis after being filtered using a 0.45 μm membrane syringe filter.

Chromatographic conditions and analysis

Utilizing UHPLC-DAD (UltiMate 3000 Dionex), the ascorbic acid concentration was assessed in accordance with a previously described procedure (Asfaw et al., 2023). The mobile phase was a combination of A - methanol (>99 %, Sigma-Aldrich, Germany) and B − 1 % aqueous acetic acid (>99 %, Sigma-Aldrich, Germany) on a reversed-phase Fortis C18 column (250 4.6 mm, 5 m; Fortis Technologies, Neston, UK). The chromatographic gradient conditions were: equilibration time from 0 to 1 min at 100 % (B), up to 3 min at 90 % (B), up to 6 min at 60 % (B), up to 10 min at 40 % (B), up to 15 min at 100 % (B), up to 20 min at 80 % (B), up to 25 min at 90 % (B), and back to equilibration time for 1 min at 100 % (B). The mobile phase flow rate, sample injection volume, column temperature, and run-time were 0.8 mL min^{-1}, 10 μL, 25 ℃, and 26 min, respectively, while analysis was done at 248, 254, 272, 278, and 450 nm. Finally, the wavelength at 278 nm, having less interference, was found to be optimum and selected for the analysis of ascorbic acid. The amounts of ascorbic acid in the extract samples were calculated from calibration equations.

Method validation

With the extracting solvent, ascorbic acid standard solutions with 11 points of calibration standards (0.5, 1, 2.5, 5, 10, 20, 40, 60, 80, 100, 150 mg/L) were made. They were then examined in triplicates. By graphing the average peak areas vs the concentrations of each standard, the calibration curve was created.

The limit of detection (LOD) and limit of quantification (LOQ) were determined from multiple measurements of the spiked (with the smallest concentration with the smallest signal close to base line or noise) blank solution (n = 7) and were calculated as three and ten times the standard deviation of the blank signals divided by the slope of the calibration equation, respectively. Using matrices-matched recovery trials, the sample preparation and analysis method's accuracy was assessed.

Recovery studies were used to test the accuracy of the procedure. These tests involved adding 20 and 40 mg/L ascorbic acid standards to a 1 g sample, followed by extraction and analysis. Seven replicate recovery trials were extracted and analyzed. The intra- and inter-day recovery experiments (n = 7 for each day) were conducted to evaluate the optimized method's intra- and inter-day precision and accuracy.

Determination of total phenolics and antioxidant activities

Total phenolic content

The TPC of the fruit extracts was determined by UV–Vis spectrophotometer (with 500 μL size quartz cuvette) using the *Folin-Ciocalteu* reagent following a method described by Asfaw et al., (2023). Briefly, 400 μL of the extract was mixed with 400 μL of Folin-Ciocalteu reagent which was diluted with methanol (1:10). After 5 min, 400 μL of 0.2 mM Na_2CO_3 solution was added to the prepared mixture. After 10 min 200 μL of 3 % $NaNO_2$ solution was added and thoroughly mixed. The mixture was left for 60 min and absorbance was measured at 765 nm. Finally, the TPC in the fruit extract was calculated from the gallic acid (mg GAE/g dry weight (d.w.), GAE – gallic acid equivalent) standard calibration curve (ranging from 50 to 400 mg/L). The analysis was done in triplicate.

Total flavonoid content

The total flavonoid contents (TFC) were measured in order to have an estimate of the flavonoid and non-flavonoid contents of the fruits from the total polyphenols determined. For TFC determination, a previously reported method (Belayneh et al., 2022) was used. Briefly, an aliquot of 200 μL of each of the fruit extract solutions of the air-dried, sun-dried, and freeze-dried samples was thoroughly mixed with 200 μL of 2 % $AlCl_3$ solution and allowed to stand for 30 min in the dark at room temperature. The absorbance of the clear-yellow color solution was measured at 417 nm. Then, the concentration of TFC (mg QE/g d.w., where QE - quercetin equivalent) in the extracts was calculated from the standard calibration curve of quercetin in the range of 50 to 500 mg/L. The analysis was done in triplicate.

DPPH radical scavenging activity

The two edible fruits and the peel were evaluated for their ability to scavenge free radicals under three different drying conditions and two different extraction techniques using the DPPH method as described by Asfaw et al., (2023). In a 50 mL brown volumetric flask, 1.9716 mg of DPPH was dissolved in methanol to create the DPPH stock solution. The absorbance reading was then corrected to 1 ± 0.02 by adding methanol or the DPPH stock solution. Exactly 2850 μL of DPPH solution was mixed with 150 μL of diluted fruit extract, vitamin C, or Trolox series standards in 5 mL amber vials. After thoroughly shaking, the mixture was left at room temperature in the dark for 30 min before the absorbance was determined at 517 nm. Finally, the percentages of the radical scavenging activities of the extracts were calculated.

ABTS radical scavenging activity

The scavenging effect of ABTS radical was measured following the method reported by Maduwanthi & Marapana, (2021) (Maduwanthi & Marapana, 2021). The reaction of an equivalent amount of 7 mM ABTS (360.234 mg of ABTS and 2.45 mM potassium persulphate) produced the ABTS radical cation ($ABTS^{\bullet+}$). Prior to usage, the mixture was kept at room temperature in the dark for 12 h. Methanol was used to dilute the ABTS solution to an absorbance of 1.0 ± 0.04 at 734 nm. In 5 mL amber vials 150 μL of fruit extracts or Trolox/ascorbic acid standards were added, then 2850 μL of ABTS solution was added. After 4 min of incubation, the absorbance at 734 nm was measured. Within the 0.05–0.90 mM range, the Trolox standard calibration curve was created. The radical scavenging effects using the percentages of the radical scavenging activities of the extracts were calculated.

Ferric reducing antioxidant power

The ferric-reducing antioxidant power (FRAP) assay was performed using a formerly described method (Maduwanthi & Marapana, 2021). In a 500 mL brown container, the fresh FRAP reagent was made by combining 250 mL of acetate buffer (300 mM, pH 3.6), 50 mL of 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), and 50 mL of 50 mM $FeCl_3{\cdot}6H_2O$. Following that, the mixture was heated to 37 ℃ and let for 10 min to react. Using 5 mL amber vials, 150 μL of fruit extract, Trolox, or ascorbic acid was added. Then, 2850 μL of FRAP solution was added. After 4 min, the absorbance was measured at 734 nm. The Trolox or ascorbic acid standard curve was constructed between 0.025 and 0.7 mM. Results were calculated from the calibration curve and are expressed as millimolarTrolox equivalent per gram (mM TE/g) fresh mass.

The half-maximum effective concentration (EC50)

The GraphPad Prism Statistical tool was used to estimate the EC50 for the DPPH, ABTS, and FRAP free radical scavenging activities of the extracts (Belayneh et al., 2022; Chen et al., 2013; Sridhar & Linton, 2019). The evaluation of the EC50 for each antioxidant parameter (using 8–9 different concentration levels)was done using non-linear regression, which is log [agonist - oxidant inhibitor conc.] in mg/mL vs. normalized response - variable slope (% of inhibition - percent of radical scavenging activities (%RSA) for each antioxidant parameter. The non-linear regression or variable slope (other than a standard slope of 1) was performed by transforming and normalizing the experimental results between 0 (basal – minima) and 100 % (maximal - top) responses that make plateaus. To estimate EC50 values with multiple-point concentration ranges, the log[agonist conc.] is the concentration that elicits a response halfway between the minimum (bottom) and maximum (top). The EC50 activities were created using 8–9 points of various concentration ranges for samples ranging from 0.05 mg/mL to 80 mg/mL and standards for ascorbic acid and trolox standards ranging from 0.025 mg/L to 500 mg/L and 25 M to 900 M, respectively.

Statistical data analysis

The results obtained in this study were expressed as mean ± standard deviation with three replicates. The analysis of variance and significant differences among the means and correlation analysis was performed with one-way ANOVA for simple one-factor analysis and two-way ANOVA for the three (types of fruits, drying conditions, and extraction methods) statistical comparisons using SPSS v24 (IBM, UK). Each instance was compared using a one-way ANOVA with Tukey's post-hoc multiple comparisons. In addition, based on the kinds of data obtained, ChemDraw (Ultra 12.0.2, CambridgeSoft, UK), Design Expert (StatEase V13), GraphPad Prism (V9, USA), and Microsoft Excel (2010) were utilized.

Results and discussion

Optimization of extraction conditions using response surface methodology

Method optimization and model validation

Method optimization. The relationships between the independent variables (extraction conditions) and responses (extraction yield) were examined based on the quadratic model (Eq. 1). This was performed to identify the best extraction conditions for DPPH antioxidant assay in terms of EC50 values, phenolics (TPC, TFC), and ascorbic acid in the studied plant samples in terms of time, temperature, solvent mixture, and fruit powder-to-solvent ratio. The quadratic models were significant ($p < 0.001$) and well-fitted the data obtained for TPC, EC50, and ascorbic acid (Supplementary files – Tables S1–S10). The adjusted and predicted determination coefficient (R^2) values of 0.9770 to 0.9997 and 0.9608 to 0.9992, respectively, were closer to each other indicating the model is significant ($p < 0.001$). As indicated from the predicted vs. actual and normal probability plots, it also shows that there were excellent correlations between the experimental and predicted values (Supplementary files Fig. S1 and S2).

Based on adequate precision, if the signal-to-noise ratio of the model is greater than 4, the model is considered desirable and can be used to navigate the design space. Adequate precision is the measure of the signal-to-noise ratio of the model. As shown in Fig. 1 and Supplementary files - Table S11, actual vs predicted data indicated that the sample linear data confirms a hypothesized distribution based on experimental data. In order to demonstrate how the anticipated data and the experimental data plotting of model terms could be helpful in selecting the right distribution of responses, this demonstrated statistical goodness of fit. This implies that the answers are considerably influenced by each model component (factor) (Almusallam et al., 2021; Belwal et al., 2016).

For the measurements of TPC, TFC, EC50, and ascorbic acid in plant samples, the predicted R^2 of all regression quadratic models and their fit statistics were in good agreement with the adjusted R^2 (the difference is ≤ 0.2, Supplementary files - Table S11). This demonstrated that the models were precise and had a good match with the chosen components and data responses. The F-values of the model were in the range of 0.23–2643.38, which implies that there is only a 0.01 % chance that an F-value of this large could occur due to noise. The signal-to-noise ratio (which is a measure of signal adequacy) of the model was in the range of 27.25–214.80 (greater than 4 is desirable), which implies that the model is significant ($p < 0.001$) (Supplementary files - Table S11). In addition when the model p-values are less than 0.05 (in this case $p \leq 0.001$, at a 99.999 confidence level = CL), the model terms are highly significant. Moreover, model p-values less than 0.01 indicated that the model terms are significant at a 99 % CL. As a result, all of the model terms are significant ($p < 0.01$) except for linear term-BC ($p = 0.23$) and quadratic term-B^2 ($p = 0.16$) in TPC, linear term-AC ($p = 0.34$), quadratic term-B^2 ($p = 0.53$), linear terms-B ($p = 0.50$), and –C ($p = 0.20$) in EC50 values (Supplementary files - Tables S3–S10). This indicated that these model terms are not attained at their optimum conditions or they are nearly to the optimum conditions, and these are not significantly affect the yields. Additionally, there are very few model terms that are non-significant at $p < 0.01$. The coefficient of variation (CV) ranging from 0.41 to 6.0 % also indicated that the designed models were a dispersion of individual measurements for their mean for the determinations of those responses (Supplementary files - Table S11). For the optimization, it was important to consider duration, temperature, methanol, and water mixture, the ratio of fruit powder to solvent, and all of these elements' interactions. As a consequence, the 15 min extraction period, 35 ℃ temperature, 75/25 % methanol–water solvent mixture, and a 1:15 (w/v) fruit powder to solvent ratio were chosen.

Model validation. The criteria chosen to process model validation of

independent and response variables were based on desirable predicted and response values as presented in Table 2. By running the optimum solution with the highest desirability, the criteria for numerical solution were analyzed to evaluate the validation of the model. Based on the outcomes, the relative error percentage ranged from 0.00 to 2.99 % (Table 2). Except for the EC50 in SCFfd (10.85 %), all anticipated and experimental values had relative errors of less than 5 %. The outcomes demonstrate that the experimental data and the projected data were in good agreement (Almusallam et al., 2021; Arruda et al., 2017; Belwal et al., 2016). Additionally, maximization (for TPC and ascorbic acid) and minimization (for EC50 values) of independent variable conditions of TPC and EC50 suggest that treatment of the CMFfd, CMPfd, and SCFfd at 15 min, 35 ℃, 75 % of methanol, and 1:15 of fruit powder-to-solvent ratio can achieve the maximum desirability function (1.00), which meets all the objectives presented in Table 2. The model fit well to the spatial influence that the independent variable had on the response, as evidenced by the non-significant value of lack of fit (F = 0.11–0.99).

The effects of extraction time (A), temperature (B), methanol concentration (C), and fruit powder-to-solvent ratio (D) on TPC and EC50 were examined in the analysis of experimental results. The quadratic equations were fitted to the coded equations with coded coefficients of the model terms for each of the responses (Eqs. 3–8). In CMFfd (Eq. (3), the model's linear terms (A, C, and D) and interaction terms (AC, BD) had positive significant ($p < 0.001$) effects, but the remaining model elements had negative significant effects. There are additional substantial model terms for the other yields, as indicated in Equations (4)–(8). As a result, the model terms are important for calculating TPC, TFC, and EC50 in plant sample samples.

For ascorbic acid, the effects of extraction time (A), temperature (B), and fruit powder-to-solvent ratio (C) were fitted with quadratic equations (Eqs. (9)–(11) for each dried fruit and peel powder extract. Strongly significant linear terms (A, B, C), as well as interaction terms (AB, BC, BDCD for CMFfd; AC, AD for CMPfd; AC, BC for SCFfd), were present for the CMFfd. However, for every quadratic term in all samples, ascorbic acid was very adversely significant ($p < 0.001$). Ascorbic acid determination in plant samples therefore depends on the model terms.

$$TPC(CMFfd) = 620.02 + 19.70A - 10.90B + 24.74C + 25.25D - 25.88AB + 11.48AC - 10.36AD - 7.13BC + 27.04BD - 12.42CD - 52.77A^2 - 30.42B^2 - 64.66C^2 - 41.31D^2 \quad (3)$$

$$TPC(CMPfd) = 672.49 + 9.07A - 11.08B - 25.12C + 22.11D + 17.40AB - 11.02AC - 25.83AD - 1.23BC - 21.25BD + 12.17CD - 86.44A^2 + 9.51B^2 - 45.48C^2 - 111.02D^2 \quad (4)$$

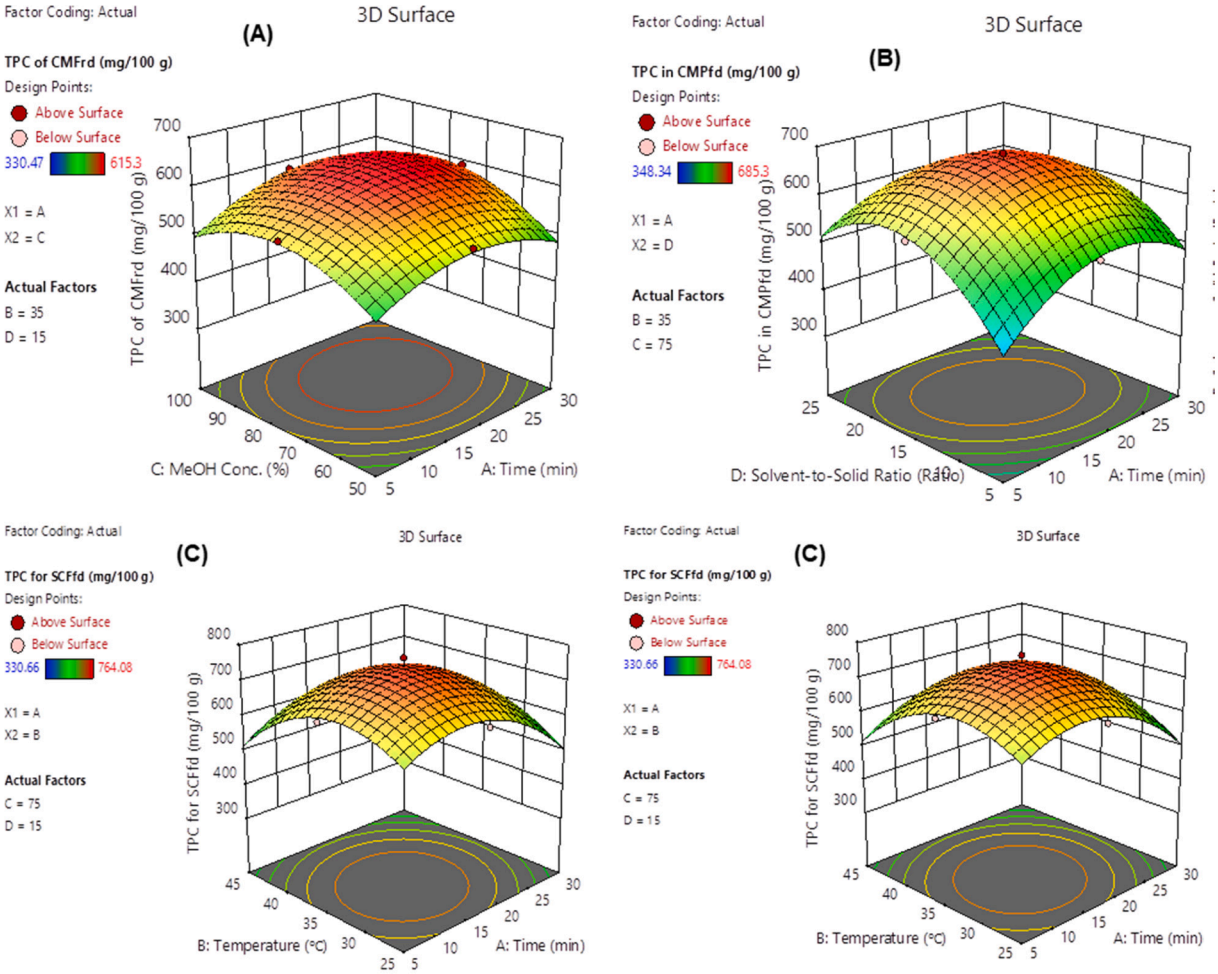

Fig. 1. Interaction effect of the different extraction conditions (time, temperature, MeOH conc., and solid-to-solvent ratio) on the measured TPC, DPPH/EC50, and AA (ascorbic acid). These 3D surface graphs show only the two specific interaction effects and responses (A = TPC for CMFfd, B = TPC for CMPfd, C = TPC for SCFfd, D = EC50 for CMFfd, E = EC50 for CMPfd, F = EC50 for SCFfd, G = ascorbic acid for CMFfd, H = ascorbic acid for CMPfd and I = ascorbic acid for SCFfd); for the rest of the graphs, it is available in the Supplementary files.

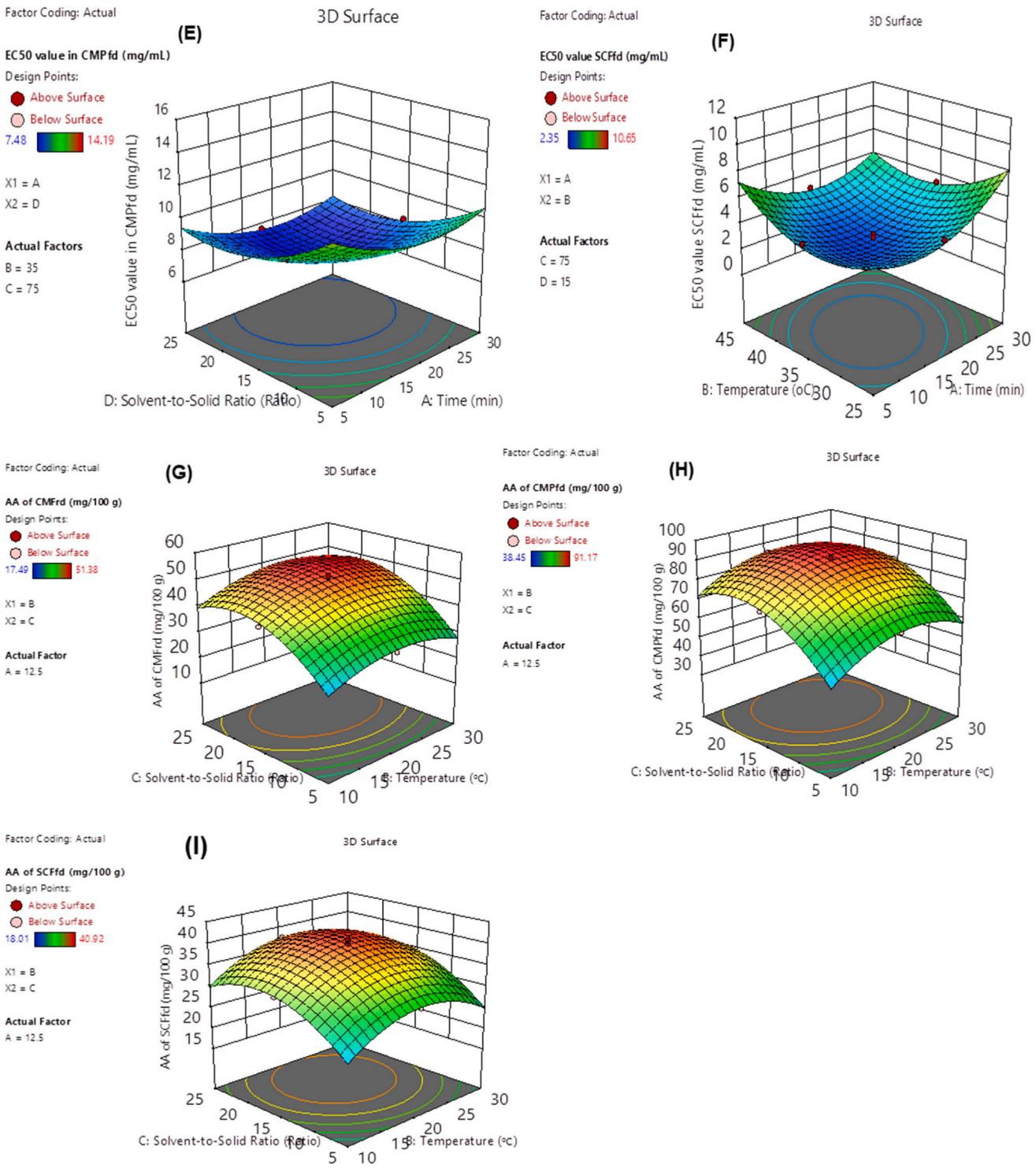

Fig. 1. (*continued*).

$$TPC(SCFfd) = 741.83 - 33.04A - 28.43B + 11.88C - 26.63D + 24.46AB - 57.17AC + 12.64AD - 8.38BC - 28.21BD - 25.93CD - 103.01A^2 - 104.70B^2 - 27.56C^2 - 26.11D^2 \quad (5)$$

$$\mathrm{EC50}(CMFfd) = 19.65 - 0.20A + 0.85B + 0.36C - 2.16D + 0.67AB - 0.39AC - 0.97AD - 0.17BC + 0.90BD + 0.28CD + 2.94A^2 + 3.68B^2 - 0.86C^2 + 4.45D^2 \quad (6)$$

Table 2
Model validation with optimum time (15 min), temperature (35 ℃), methanol conc., (75 %), and fruit powder-to-solvent ratio (1:15) conditions of UAE on the TPC, EC50, and ascorbic acid contents of CMFrd, CMPfd, and SCFfd.

Predicted Vs Experimental	Time (min)	Temperature (℃)	Methanol Conc. (%)	Powder-to-solvent Ratio	*C. medica*fruit (mg/100 g)		*C. medica*fruit peel (mg/100 g)		*Z.spina-christi* fruit (mg/100 g)	
					TPC	EC50	TPC	EC50	TPC	EC50
Predicted	17.5	35	75	15	615.3	18.69	685.87	7.44	767.71	2.12
Experimental	15	35	75	15	615.3 ± 5.68	19.40 ± 0.84	685.30 ± 4.24	7.29 ± 0.61	764.08 ± 25.79	2.35 ± 0.09
Relative error (%)	14.29	0.000	0.000	0.000	0.000	3.80	0.08	2.02	0.47	10.85
					1 % aqueous acetic acid UAE extract of ascorbic acid (mg/100 g)					
					CMFfd		CMPfd		SCFfd	
Predicted	12.5	25	–	15	52.61		94.24		40.98	
Experimental	10	25	–	15	51.38 ± 1.44		91.17 ± 2.57		40.92 ± 2.24	
Relative error (%)	20	0	–	0	2.34		3.26		0.15	

$$\mathrm{EC50}(CMPfd) = 7.58 - 0.63A + 0.18B + 0.86C - 1.25D - 0.38AB + 0.03AC + 0.07AD - 0.55BC - 0.49BD - 0.05CD + 1.24A^2 + 0.04B^2 + 0.76C^2 + 1.27D^2 \quad (7)$$

The effect of methanol concentration and extraction time, the effect of temperature, and methanol on the contents of TPC, TFC, antioxidants, and ascorbic acid are shown in Fig. 1. Strong positive effects of time and solvent-to-solid, and the effects of temperature and fruit powder-to-solvent ratio on the contents of antioxidants (DPPH and EC50) was observed (Fig. 1). Based on the experimental results, the optimal predicted extraction time was 17.5 min, the temperature was 35 ℃, methanol concentration in water was 75 % and the fruit powder-to-solvent ratio was 1:15. There were no significant variations between the predicted extraction conditions and experimental conditions in all of the variables.

$$\mathrm{EC50}\,(SCFfd) = 2.79 + 0.60A + 0.05B - 0.10C + 0.58D - 1.02AB + 1.34AC + 0.26AD + 0.09BC + 0.65BD + 0.96CD + 1.96A^2 + 1.87B^2 + 0.38C^2 - 0.09D^2 \quad (8)$$

$$\text{ascorbic acid}(CMFfd) = 49.85 + 3.52A + 2.41B + 8.12 - 1.38AB + 1.67AC + 0.84BC - 4.50A^2 - 5.75B^2 - 9.66C^2 \quad (9)$$

$$\text{ascorbic acid}(CMPfd) = 89.90 + 7.13A + 3.09B + 10.23C - 1.21AB + 2.66AC - 0.58BC - 6.37A^2 - 11.06B^2 - 14.56C^2 \quad (10)$$

$$\text{ascorbic acid}(SCFfd) = 39.81 + 2.47A + 1.16B + 3.68C - 0.62AB + 1.23AC + 0.36BC - 3.55A^2 - 6.07B^2 - 5.93C^2 \quad (11)$$

The 3D surface graphs (Fig. 1, and Supplementary files - Fig. S3-S4) of the analyzed parameters showed that the strong positive effect of extraction temperature, time, powder-to-solvent ratio, and solvent mixture were found to be significant ($p < 0.001$) for all of the responses. With a highly significant ($p < 0.0001$, Supplementary files Tables S3–S10) impact, the 3D surface graphs of TPC, DPPH/EC50, and ascorbic acid vary over time as a function of methanol concentration and temperature as a function of fruit powder to solvent ratio, shifting to the maximum and minimum yields (Fig. 1). The TPC TFC and ascorbic acid extraction yield for CMPfd increased from 330.47 to 685 mg/100 g, 24.26 to 30.39 mg/100 g, and 38.45 to 91.17 mg/100 g, respectively, with increasing time (5 to 17.5 min) and methanol concentration (45 to 75 %), before declining. With increased values of temperature and the fruit powder-to-solvent ratio from 25 to 35 °C and from 5:1 to 15:1, respectively, the EC50 value of CMPfd dropped to the maximal inhibitory concentrations (7.480.73 mg/100 g) (Fig. 1B). Similar trends were observed in the other parameters and dried fruits (Supplementary files - Fig. S3-S4). The fruits of different species reported in different countries (Almusallam et al., 2021; Belwal et al., 2016; Ordóñez-Santos et al., 2015; Prakash Maran et al., 2017) have similar results on TPC, TFC, and DPPH for those and other factors such as the power of ultrasound.

In particular cases, the interaction effects of extraction time with water in methanol (75:25 %) and temperature with methanol in water have higher significant effects on all of the extraction yields. The extraction yields of TPC, ascorbic acid, and *in vitro* antioxidant activities increased with increasing extraction time, temperature, methanol ratio in water, and fruit powder-to-solvent ratio from 5 to 15 min, 20 to 35 ℃, 45 to 75 %, and 1:5 to 1:15, respectively (Fig. 1). On the other hand, the EC50 values decreased up to the optimum conditions (15 min, 35 ℃, and 1:15 ratio) and then increased after the optimum conditions (15 to 25 min, 35 to 45 ℃, and 75 to 100 % methanol) (Fig. 1).

The central 3D surface graphs displayed symmetrical surfaces with the highest TPC and ascorbic acid responses in all fruit samples. Since the surfaces would have smaller values of the reactions as it is closer to the center, a similar shape could be obtained for the lowest responses of EC50 values for all of the fruits and peel powder extracts. The findings suggest that duration, temperature, methanol concentration, and the ratio of fruit powder to solvent are best combined to produce maximum (TPC and ascorbic acid) and minimum (EC50) values. Some of the 3D surface graphs such as effects of time with methanol concentration, methanol concentration with fruit powder-to-solvent ratio, and temperature with methanol concentration on EC50 in CMFfd showed saddle or minimum surfaces, suggested that the responses from the region's center would decline. In contrast, some of the 3D surface graphs such as time with fruit powder-to-solvent ratio, and temperature with fruit powder-to-solvent ratio on TPC in CMPfd, and time with methanol concentration on EC50 in CMPfd showed a rising or sloping ridge, which indicated that TPC and ascorbic acid levels reached their highest and lowest points outside of the design spaces (Herrera-Pool et al., 2021). The interaction effects of time with temperature and temperature with fruit powder-to-solvent ratio on EC50 values in CMPfd showed a stationary ridge, which indicated that the minimum or maximum values are remote from the center of the region. The other orientations (Fig. 1) of the graphs showed good interactions between two factors at a time.

These experimental variables that impacted the parameters that were obtained were consistent with certain recently published research (Almusallam et al., 2021; Belwal et al., 2016; Ordóñez-Santos et al.,

Table 3
Total phenolic, total flavonoid, and ascorbic acid contents of *C. medica* fruits, *C. medica* fruits peel, and *Z.spina-christi* fruit under different drying conditions.

Fruits and Drying Methods	Methanol Soxhlet extract		75 % methanol UAE (mg/100 g)		1 % aq. AcOH
	TPC (mgGAE/100 g)	TFC (mgQE/100 g)	TPC (mgGAE/100 g)	TFC (mgQE/100 g)	ascorbic acid UAE(mg/100 g)
CMFfd	255.46 ± 1.59^{d}	127.70 ± 17.25^{b}	428.22 ± 2.46^{a}	213.11 ± 17.25^{b}	51.38 ± 1.44^{e}
CMFrd	264.55 ± 3.56^{d}	142.26 ± 3.56^{b}	615.3 ± 5.68	284.43 ± 6.64^{b}	32.50 ± 1.44^{c}
CMFsd	239.84 ± 65.22^{c}	82.55 ± 65.22^{a}	393.66 ± 2.08	265.48 ± 10.80^{c}	8.67 ± 1.39^{a}
CMPfd	334.98 ± 20.84^{e}	131.76 ± 11.16^{b}	685.30 ± 4.24	334.98 ± 20.83^{d}	91.17 ± 2.57^{f}
CMPrd	201.98 ± 14.70^{b}	101.41 ± 2.34^{b}	413.55 ± 4.00^{a}	201.98 ± 14.70^{b}	54.16 ± 1.22^{e}
CMPsd	72.89 ± 2.04^{a}	79.39 ± 4.70^{a}	253.03 ± 6.31	101.41 ± 6.31^{a}	13.43 ± 3.27^{b}
SCFfd	485.98 ± 9.18 ^{g}	290.67 ± 5.19^{e}	764.08 ± 25.79^{b}	485.98 ± 9.19^{f}	40.92 ± 2.24^{d}
SCFrd	385.5 ± 11.70^{f}	242.53 ± 11.70^{d}	728.99 ± 5.51^{b}	385.51 ± 11.74^{e}	32.08 ± 1.79^{c}
SCFsd	237.72 ± 18.64^{c}	218.10 ± 58.63^{c}	594.22 ± 18.03	298.27 ± 12.48^{b}	14.64 ± 1.07^{b}

CMFfd = *Citrus medic*a fruit freeze-dried, CMPfd = *Citrus medica* fruit peel freeze-dried, SCFfd = *Z. spina-christi* fruit freeze-dried, and 1 % aq. AcOH ascorbic acid = 1 % of aqueous acetic acid and ascorbic acid. Mean values with different superscript letters are significantly different at $p \leq 0.05$.

2015; Prakash Maran et al., 2017). The response variables were all significantly impacted by each of the variables and their interactions (Fig. 1 and Table 2). Overall, the experimental design (FC-CCD) was successful in examining the concentrations of TPC, EC50 values, and ascorbic acid in CMFfd, CMPfd, and SCFfd extracts, as seen from the 3D surface graphs (Fig. 1).

Studies have been reported for other plant species, where the optimization of factors such as extraction temperature, power of ultrasound, extraction time, and the solid–liquid ratio increases the extraction efficiency of bioactive components. For instance, extraction conditions in the determination of total anthocyanin, phenolic, and flavonoid contents of *Nephelium lappaceum* L. fruit peel (Prakash Maran et al., 2017) have been optimized and reported. In addition, optimizing extraction temperature, time, and methanol concentration has been found to maximize the recovery of TPC in *Phoenix dactylifera* (L.) (Almusallam et al., 2021), ultrasound power, time, and temperature in the peach palm (*Bactris gasipaes*) fruit by-products (Ordóñez-Santos et al., 2015). Extraction optimization of TPC and antioxidant potential of *Berberis asiatica* fruits (Belwal et al., 2016), and the effects of extraction time, temperature, and antioxidant activities of seed extract (Choi et al., 2022) have been reported. The effects of time, temperature, and ethanol concentration on the antioxidant potential of *Nypa fruticans* Wurmb. (Choi et al., 2022) have also been studied. The optimized conditions of UAE for the currently studied fruits have similar effects on the extraction yields of TPC, TFC, and, ascorbic acids, and the antioxidant potentials of the selected fruits. Therefore, these showed that optimizing the main factors that can significantly affect the contents of phenolic compounds and antioxidant properties of the studied plants is appropriate.

Comparison between ultrasonic assisted and soxhlet extraction

The TPC values varied from 72.89 ± 2.04 mg GAE/100 g (CMPsd) to 485.98 ± 9.18 mg GAE/100 g (SCFfd) using the soxhlet extraction method while from 253.03 ± 6.31 mg GAE/100 g (CMPsd) to 764.08 ± 25.79 (SCFfd) using the UAE method (Table 3). The TPC was found in the decreasing orders of SCFfd > SCFrd > CMPfd > CMFrd > CMFfd > CMFsd > SCFsd > CMPrd > CMPsd for both the soxhlet and UAE methods, respectively. The same fashions were observed for TFC. As observed from this, the UAE provided significantly higher TPC than the soxhlet extracts.

The *C. medica* (its peel) and *Z. spina-christi* fruit extracts in soxhlet methanol and UAE methanol–water mixture exhibited the best inhibitory effect against DPPH, ABTS, and FRAP radicals. Fruit soxhlet methanol extracts had radical scavenging capacities against EC50 values of DPPH that ranged from 3.49 ± 0.40 mg/mL to 40.61 ± 1.13 mg/mL. On the other hand, the scavenging activities of UAE-optimized extracts of fruits against EC50 values of DPPH ranged from 2.35 ± 0.09 mg/mL to 26.08 ± 2.86 mg/mL. The EC50 values in DPPH scavenging activities of UAE optimized extraction method were significantly lower than that of soxhlet extracts. Therefore, the UAE method was better than that of the soxhlet extractor.

The presence of water in the UAE extraction mixture, compared to the pure methanol used with Soxhlet, acts as the driving force for a higher diffusion of solvents into the fruit powders that can increase the mass transfer of the target analytes (mostly polar bioactive compounds) from the powder to the solution (Herrera-Pool et al., 2021). At 25 % of water contents, the maximum recoveries of TPC, EC50, and ascorbic acid were improved because as the polarity increased, the acoustic cavitation (mass transfer) for the formation of bubbles increased (Herrera-Pool et al., 2021), and enhanced extraction efficiency (Belwal et al., 2016).

Comparison among drying conditions for ascorbic acid

The ascorbic acid content of *C. medica* fruit ranged from 8.67 mg GAE/100 g with sun drying (CMFsd) to 51.38 mg GAE/100 g with freeze drying (CMFfd) (Table 3). From the fruit peel of *C. medica*, ascorbic acid in CMPsd (13.43 ± 3.27 mg/100 g) was the lowest, and in CMPfd (91.17 ± 2.57 mg/100 g) was the highest. The amount of ascorbic acid in *Z. spina-christi* fruits, SCFfd (40.92 ± 2.24 mg/100 g) was the highest.

Except for the *C. medica* peels, which exhibited similarities between the sun-dried and room-dried samples, all three drying conditions examined for both *C. medica* and *Z. spina-christi* fruits and peels yielded significantly different ($p < 0.05$) concentrations of ascorbic acid. In each of these instances, freeze-drying delivered a greater quantity of ascorbic acid than the alternatives. Thus, the VFD method preserves more ascorbic acid than other treatment methods. The amounts of ascorbic acid in samples treated by VFD were significantly ($p < 0.05$) different from those of SD and RD. Recently, evaluations and reports on the impacts of drying techniques on various food matrices have been made (Calvo-Calvo-Brenes and ÓHare, 2020), drying by hot air, microwave, sun, shade, and freezing on phenolic compounds and antioxidant properties in lemon myrtle (Oyarzún et al., 2020). The phytochemical and antioxidant activity of unmatured citrus fruits have been observed to be affected by the sun, hot air, and freeze-drying techniques (Sun et al., 2015). These reported methods showed that freeze-drying significantly retained the contents of phenolic compounds and

Table 4
Comparison of antioxidant potentials (EC50) of the fruits and peels under different drying conditions and extraction techniques used.

Dried Fruits	EC50 for Soxhlet extract (mg/mL)	EC50 for UAE (mg/mL)
CMFfd	31.89 ± 0.90^{c}	19.40 ± 0.84^{d}
CMFrd	36.23 ± 0.88^{d}	21.96 ± 1.51^{c}
CMFsd	40.61 ± 1.13^{b}	25.60 ± 1.96^{b}
CMPfd	10.02 ± 0.55^{e}	7.29 ± 0.61^{e}
CMPrd	25.54 ± 0.86	7.48 ± 0.73^{f}
CMPsd	39.23 ± 1.61^{b}	26.08 ± 2.86^{b}
SCFfd	3.49 ± 0.40^{a}	2.35 ± 0.09^{a}
SCFrd	3.62 ± 0.54^{a}	2.45 ± 0.30^{a}
SCFsd	4.77 ± 0.19^{f}	2.89 ± 0.21 ^{g}

*EC50 (half-maximum effective concentration) values for the references Trolox and ascorbic acid were 0.33 ± 0.01 and 0.29 ± 0.03 mg/mL, respectively. Mean values with different superscript letters in the same column are significantly different at $p \leq 0.05$. CMFfd = Citrus medica fruit freeze-dried, CMPfd = Citrus medica fruit peel freeze-dried, SCFfd = *Z. spina-christi* fruit freeze-dried. UAE is ultrasound-assisted extraction.

Table 5
Two-way ANOVA for EC50 of the fruits and peels under different drying conditions and extraction methods used.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2722.808	8	340.351	14.02278	0.000583	3.438101
Columns	354.6672	1	354.6672	14.61263	0.005071	5.317655
Error	194.1703	8	24.27128			
Total	3271.645	17				

antioxidant properties of the studied plants than other drying methods. For the same plant material, the highest concentration of ascorbic acid was measured in freeze-dried samples, while the lowest was in sun-dried samples. This showed that the contents of ascorbic acid in fruits were well preserved during the freeze-drying process. Therefore, freeze-drying is an appropriate sample pretreatment in the analysis of ascorbic acid in the fruits and peels of the studied plants.

Comparison among drying conditions for total phenolic content and antioxidant assays

The types of assays employed and the fruit samples were compared using statistical significance tests ($p = 0.05$) (Tables 3). From the optimized UAE extracted fruits and *C. medica* peel, there were no significant differences between CMFfd and CMPrd, and SCFfd and SCFrd for TPC. Similarly, there were no significant differences in the contents of TFC between CMFfd, and CMPrd, and CMFrd and SCFsd. However, there were significant differences between CMPfd and CMPrd, CMPfd and CMPsd, CMFrd and CMFsd, and CMPrd and SCFsd for TPC. The same significant differences were exhibited against antioxidant activities (Table 4). These significant variations indicated that drying methods differently affect the compositions of phytochemicals and antioxidant potentials of the fruits (Oyarzún et al., 2020) (Table 5).

RSM-optimized methanol–water mixture (75:25) extracts of SD, RD, and VFD fruit powders against DPPH assay were performed (Table 4, Fig. 3). The radical scavenging potentials of *C. medica* fruit extracts against EC50 values of DPPH ranged from 19.40 ± 0.84 mg/mL (CMFfd – maximum) to 25.60 ± 1.96 mg/mL (CMFsd – minimum). The antioxidant potentials of *C. medica* fruit peel and *Z. spina-christi* fruit extracts ranged from 7.29 ± 0.61 mg/mL (CMPfd) to 26.08 ± 2.86 mg/mL (CMPsd - minimum) and 2.35 ± 0.09 mg/mL (SCFfd - maximum) to 2.89 ± 0.21 mg/mL (SCFsd). This showed that the EC50 values of freeze-dried were significantly lower than other drying methods. The EC50 value of SCFfd was significantly ($p \leq 0.05$) higher than the SCFrd and SCFsd methods. The same is true for CMFfd and CMPfd which were significantly higher than room and sun-dried methods. As indicated from these results (Table 4), higher EC50 values were recorded in SCFfd, CMFfd, and CMPfd samples compared with that of the other respective dried powder extracts. Therefore, drying methods under different conditions significantly affected the scavenging ability of the fruits and the peel.

Therefore, freeze-drying, generally, provided higher TPC, TFC and DPPH, ABTS, and FRAP scavenging abilities that is due to the protection of light, air, and moisture-sensitive bioactive components (Oyarzún et al., 2020). Therefore, according to this study, the VFD method was a suitable treatment condition for the analysis of TPC, TFC, and antioxidant potential assays in these fruits.

Ascorbic acid determination by UHPLC

Extraction conditions of ascorbic acid

In this study, a new method involving 1 % aqueous acetic acid as solvent was used for the UAE and UHPLC analysis of ascorbic acid. The dried fruit samples were extracted with the UAE system, involving the optimized conditions concerning time 10 min, temperature 25 ℃, and fruit powder-to-solvent ratio 1:15. The recovery of the method was good enough to analyze the amount of ascorbic acid in fruits (Table 3). After extraction, the analysis using UHPLC-DAD was conducted within 24 h as it has also been reported by Chebrolu et al. (2012) for the grapefruits (48 h).

Cotruț & Bădulescu, (2016) reported a method for ascorbic acid from carrots, beet, and cherry tomatoes using *meta*-phosphoric acid as an extraction solvent. The author has reported a different method using 9 % *meta*-phosphoric acid and 3 % citric acid as a solvent in the extraction of ascorbic acid. Inhaling *meta*-phosphoric acid can result in burns to the skin as well as corrosive lesions to the upper respiratory tract and lungs (https://pubchem.ncbi.nlm.nih.gov/compound/Metaphosphoric-acid, access date on April 2023). Therefore, compared to the use of *meta*-phosphoric acid as an extraction solvent, the optimized method involving acetic acid, in this study, can be considered more environmentally friendly.

UHPLC determination of ascorbic acid

UHPLC method of analysis of L-ascorbic acid in different fruits was developed and optimized relative to *meta*-phosphoric acid (mPA) (Chebrolu et al., 2012), and the phosphoric and acetic acid mixes to avoid autoxidation of ascorbic acid at high pH (Melfi et al., 2018). The extracted samples were analyzed within 24 h, after this time the amount of ascorbic acid declined significantly, and new peaks were detected on the chromatogram due to the decomposition of ascorbic acid to other substances, such as dehydroascorbic acid, isoascorbic acid, and dehydroisoascorbic acid (Boonpangrak et al., 2016). For the detection of ascorbic acid using UHPLC-DAD, after scanning from 200 to 600 nm the maximum wavelengths 230, 248, 254, 272, and 278 nm were selected (Fig. 2 (A-C)). The optimized maximum wavelength, that the maximum amounts of ascorbic acid obtained, was 278 nm (Fig. 2(A-C)). As a result, 278 nm was selected as the optimal wavelength for further study (Table 3).

Method performance characteristics

The method provided good linearity ($r^2 = 0.9994$) in the concentration range of 0.50 to 150 mg/L (Fig. 2). The limit of detection (LOD) and the limit of quantitation (LOQ) values were calculated from the standard deviation of the repeated measured values of spiked blanks. The average ($n = 7$) of LOD, LOQ, and recovery were 29.01 ± 0.79 μg/100 g, 96.71 ± 2.63 μg/100 g, and 95.54 ± 4.33 %, across the different plant samples, respectively. The range of recoveries obtained from three different days of experiments ($n = 7$ for each) was 89.74–105.02 % indicating the method has shown good repeatability, reproducibility, sensitivity, and accuracy. In addition, since the extraction solvent was only 1 % aqueous acetic acid, the extraction method was green and cheap relative to the other reported methods (Chebrolu et al., 2012; Orsavová et al., 2019).

As shown in the chromatogram (Fig. 2), selective ($Rs > 1.5$ - according to AOAC requirement) and specific (100 % of its selectivity – no other peaks interfere with ascorbic acid) detection of ascorbic acid were performed using UHPLC-DAD ($Rs \geq 2.7$).

The concentration of ascorbic acid in the plant samples

Citrus fruit species are known as the sources of vitamin C and other chemical components including essential oils (Chhikara et al., 2018).

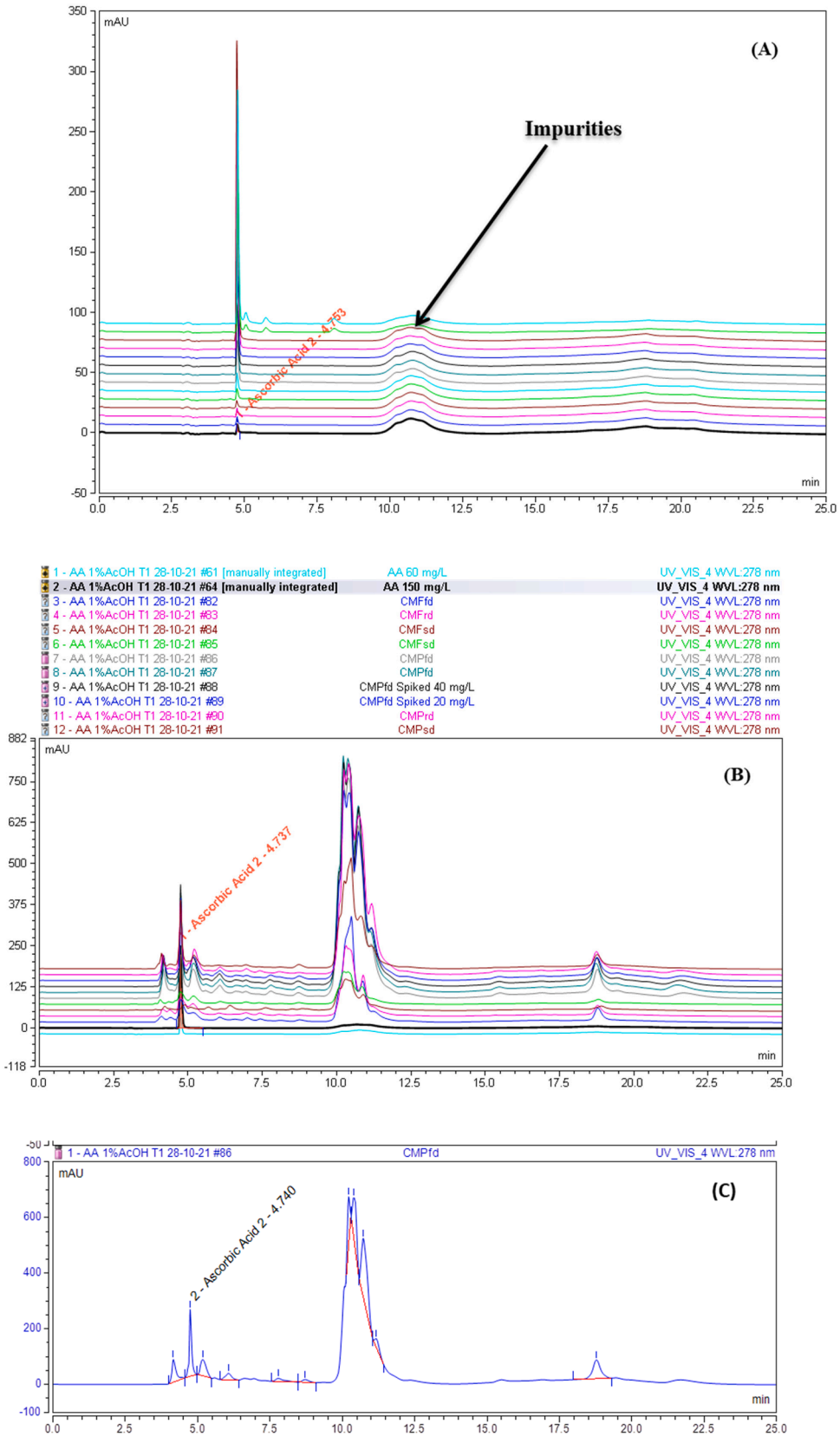

Fig. 2. Chromatograms of L-ascorbic acid (0.5–150 mg/L) standards (A), the overlaid chromatogram of samples with standards (B), and chromatogram of sample (C) at λ-max of 248 and 278 nm. Other chromatograms are available in Supplementary files – Fig. E.2.

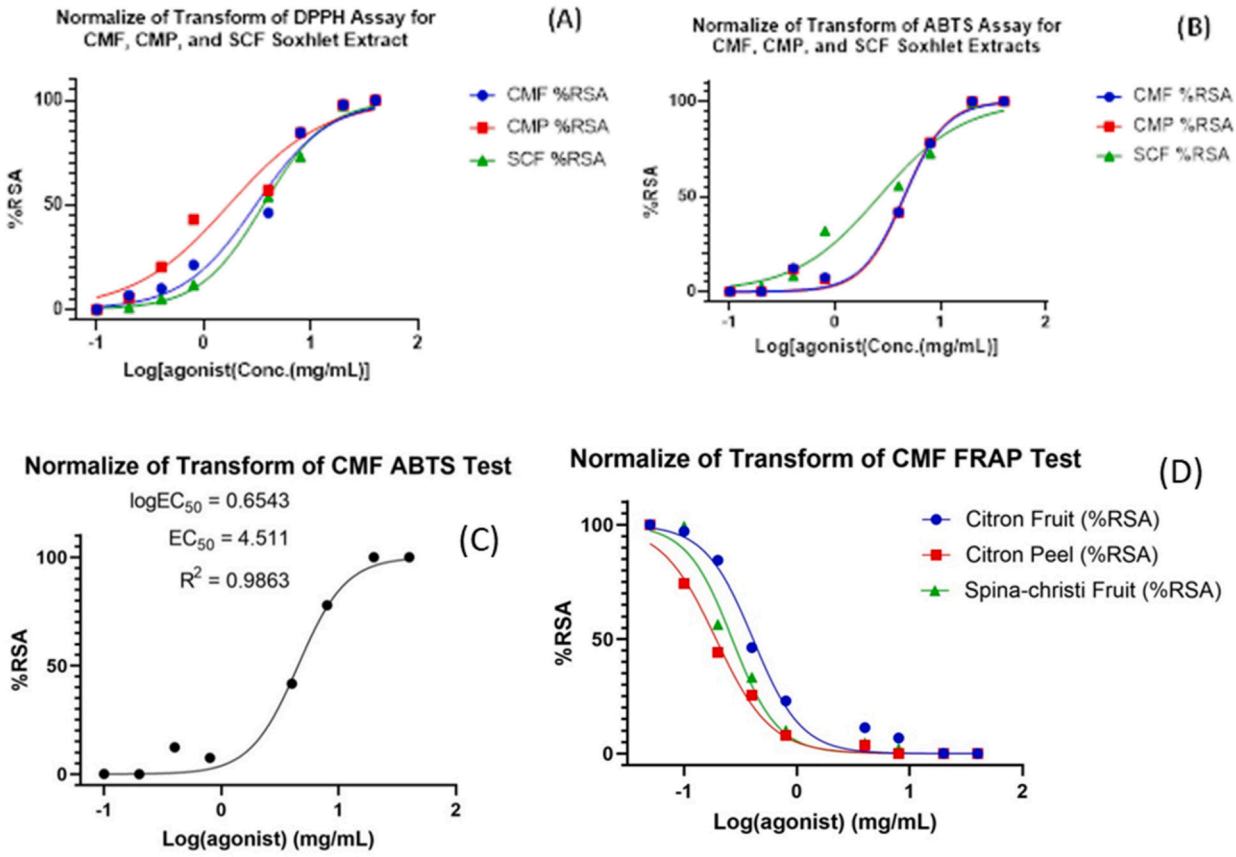

Fig. 3. Non-linear curve-fitting comparison of antioxidant activities of different concentrations of extract of: (A) DPPH assay, (B) ABTS assay, (C) sample calculation steps from ABTS assay for EC50, (D) FRAP assay, (E) DPPH assay for dried *C. medica* fruit powders of UAE after optimization, (F) DPPH assay for *C. medica* fruit peel powders of UAE after optimization, (G) DPPH assay for *Z. spina-christi* fruit powders of UAE after optimization, (H) DPPH assay for all fruit and peel UAE after optimization, (I) DPPH assay for Trolox standard, and (J) DPPH assay for ascorbic acid standard. The UV–vis spectrophotometric detections of DPPH, ABTS, and FRAP at different levels of concentration at 517, 734, and 593 nm, respectively.

The ascorbic acid in fruits varied from 40.92 ± 2.24 mg/100 g of freeze-dried *Z. spina-christi* fruit to 91.17 ± 2.57 mg/100 g of freeze-dried *C. medica* fruit peel (Table 3). On the other hand, the different freeze-dried plant samples exhibited different ascorbic acid contents, whereas peels of *C. medica* contained higher ascorbic acid followed by its fruits (51.98 mg/100 g) (Granato et al., 2016). Comparatively, the ascorbic acid content of the examined *C. medica* fruit and peel was higher than that of peeled lemons (47.9 ± 4.7 mg/100 g), oranges (49.7 ± 4.9 mg/100 g), grapes (35.1 ± 3.5 mg/100 g), orange peels (59.6 ± 5.2 mg/100 g), and grape peels (43.8 ± 4.7 mg/100 g) (Gorinstein et al., 2001). Therefore, the high ascorbic acid content will have a significant contribution to the antioxidant activities of the plant materials (Orsavová et al., 2019).

In general, the difference in concentrations of ascorbic acid in plants is strongly correlated with growing climatic conditions, soil type, and genetic variations (Orsavová et al., 2019). In addition to these, it depends on the method used to determine the contents of ascorbic acid in fruits. Unfortunately, there are limited reported studies in Ethiopia on these plant fruits and the use of this comparative method.

Antioxidant Activities, phenolics and flavonoid contents

Total phenolic contents

The obtained TPC of studied plants ranged from 253.03 ± 6.31 mg/100 g to 764.08 ± 25.79 mg/100 g (Table 3). The fruit and peel of the *C. medica* species under study contained more TPC than the reported levels of 164 ± 10.3 mg/100 g for peeled lemons, 190 ± 10.6 mg/100 g for lemon peels, 154 ± 10.2 mg/100 g for peeled oranges, 179 ± 10.5 mg/100 g for orange peels, 135 ± 10.1 mg/100 g for peeled grapes, and 155 ± 10.3 mg/100 g for grape peels (Gorinstein et al., 2001). Similarly, the current TPC was higher than the reported values of matured *C. medica* fruit (109.4 ± 2.9 mg/100 g) (Menichini et al., 2011). A recent study conducted by Alhakmani et al., (2014) on Z. spina-christi fruit extract from Oman revealed a TPC of 2462 ± 83 mg of GAE/100 g, which is significantly higher than the current study's findings. The high TPC of the fruits and the peel may be due to the significant contribution of the drying and extraction conditions (Orsavová et al., 2019).

Total flavonoid contents

The aluminum chloride assay method of TFC was found to be 213.11 ± 17.25 mg/100 g for *C. medica* fruit, 334.98 ± 20.83 mg/100 g for *C. medica* fruit peel, and 485.98 ± 9.19 mg/100 g for Z. spina-christi fruit (Table 3). The peel of *C. medica* fruit contains higher amounts of TFC as compared to the edible part of the fruit (Gorinstein et al., 2001). The higher amounts of TFC were obtained as compared to the reported amounts of lemons (47.63 ± 1.95 mg/100 g), oranges (11.82 ± 3.26 mg/100 g), and grapefruits (5.07 ± 0.82 mg/100 g) (Chun et al., 2005). This indicated that the studied fruits have high amounts of TFC and are affected by drying and extraction conditions.

DPPH radical scavenging activity

The fruit samples extracted with a 75:25 % methanol/water mixture were assessed using a widely used *in vitro* DPPH assay method. The DPPH values were found to be 175.47 ± 2.43 TEAC (mM/100 g dw) for extracts of *C. medica* fruit, and 248.63 ± 5.52 TEAC (mM/100 g dw) for *C. medica*peel (Table 4). The DPPH activities of fruit extracts were also

expressed as ascorbic acid equivalence antioxidant activities. Similar results were reported in these fruits such as on fruits and peels of *citrus* and *Z. spina-christi* and jujube fruits (Asfaw, Tadesse, et al., 2023; Taghipour et al., 2020). This indicated that the studied fruits have high antioxidant activities and are affected by drying and extraction conditions.

ABTS radical scavenging activity

The fruit-extracted samples were evaluated against ABTS assay methods (Table 4) based on both TEAC and ascorbic acid equivalence antioxidant activities (mM/100 g dw). Trolox and ascorbic acid standard curves were linear in the range of 0.025 to 0.9 mM and 0.025 to 500 mg/L, respectively, and good linearity was obtained ($r^2 \geq 0.99$). The ABTS values were found to be 167.15 ± 3.2 TEAC (mM/100 g dw) for extracts of *C. medica* fruit, and 256.67 ± 6.98 TEAC (mM/100 g dw) for *C. medica* peel. The ABTS values were found to be 399.60 ± 2.75 TEAC (mM/100 g dw) for extracts of *Z. spina-christi* fruit which have closer values to the previously reported values (Belayneh et al., 2022). The ABTS assay is more related to the TEAC assay than ascorbic acid (Belwal et al., 2016). The inhibition activities were recorded above 90 % (≥90 % RSA). The % RSA depends on the concentrations of DPPH assays, the pH of the solution, and the type of solvent used (Herrera-Pool et al., 2021). This indicated that the studied fruits have high antioxidant activities and are affected by drying and extraction conditions.

Ferric reducing antioxidant power

The extracts of fruit samples were evaluated against commonly accepted *in vitro* FRAP assays (Table 4). Trolox and ascorbic acid standard curves were linear in the range 0.025 and 0.7 mM and 0.025 to 500 mg/L, respectively, and good linearity was obtained ($r^2 \geq 0.99$). The FRAP assay was found to be 765.83 ± 22.08 TEAC (mM/100 g dw) for extracts of *C. medica* fruit, and 358.67 ± 19.89 TEAC (mM/100 g dw) for *C. medica* peel. The current FRAP activities of *Z. spina-christi* fruit have closer values to the previously reported values (Belayneh et al., 2022). The FRAP assay is more related to the ascorbic acid assay capacity than the Trolox equivalent (Belwal et al., 2016). This indicated that the studied fruits have high antioxidant activities and are affected by drying and extraction conditions.

Half-maximum effective concentrations (EC50) of fruits and peel

Using the GraphPad Prism statistical tool, the EC50 values of fruit extracts were calculated for the DPPH, ABTS, and FRAP assays. Fig. 3 shows the 8 to 9 concentration levels transformed and normalized graphs in comparison to the Trolox and ascorbic acid standards. The model was created with standards as a starting point, and it worked well with actual samples to predict EC50 values within specific maximum and minimum point ranges using the provided formula (Belayneh et al., 2022; Chen et al., 2013; Sridhar & Linton, 2019). In the DPPH assay, the lowest EC50 value of fruits was recorded for *Z. spina-christi* (2.35 ± 0.09 mg/mL) while the highest was for *C. medica* fruit (19.40 ± 0.84 mg/mL) ((Table 4)). Previously, the EC50 values of various fruits were reported as 3.68 mg/mL for DPPH, 6.07 mg/mL for ABTS, and 15.33 mg/mL for FRAP (Belayneh et al., 2022), which are higher (weaker antioxidant potential) than the current study.

In addition to the EC50 values evaluated against DPPH in Table 4, EC50 was also evaluated against ABTS and FRAP assays. The EC50 values of the fruits against ABTS were found to be the highest at 24.51 ± 0.89 mg/mL (weak antioxidant potential) and lowest at 14.03 ± 0.07 mg/mL (strong antioxidant potential). In the case of the FRAP test, the EC50 values of the highest was 25.41 ± 0.01 mg/mL and the lowest 18.19 ± 0.00 mg/mL. These values were higher as compared with reported values of 3.68 mg/mL in DPPH to 15.48 in FRAP assays for jujube species. This indicates that the studied fruits have high antioxidant potential to benefit human health.

Wild fruits, such as *C. medica* and *Z. spina-christi*, have great potential for human nutrition and health benefits from their antioxidant properties. These fruit species contained significant amounts of ascorbic acid and polyphenols as well as strong antioxidant potentials. The high levels of ascorbic acid, polyphenolic compounds, and antioxidants in citron and Christ's thorn fruits have implications in biotechnology and food science. These fruits can be used to develop functional foods and supplements, providing additional health benefits and antioxidant properties (Cerdá-Bernad et al., 2023). They can potentially replace synthetic antioxidants, improving food quality and extending shelf life. Extracts from these fruits can be used to develop nutraceuticals with concentrated levels of ascorbic acid and polyphenols, supporting various body functions. Overall, these fruits have applications in functional foods, antioxidant additives, nutraceuticals, biomedical products, and agricultural and nutritional practices. The quadratic models optimized in this study can be used in the analysis of polyphenols, EC50, and ascorbic acid in different food matrices.

The research Hypothesis is accepted: The acceptance of the research hypothesis is substantiated by the finding that the examined wild edible fruits indeed play a pivotal role as abundant sources of both ascorbic acid and antioxidants, thereby positively impacting human health. Moreover, it was observed that the composition of these fruits was affected by various factors, including drying conditions and other independent variables, throughout the extraction and analysis processes.

Conclusion

This study showed drying conditions and extraction variables for the fruits and peels of *C. medica* and *Z. spina-christi* can significantly ($p \leq 0.05$) affect the ascorbic acid, polyphenols, and antioxidant potentials of fruits. Out of the drying methods, freeze-drying significantly maintained these components. The fruit powder-to-solvent ratio, temperature, and methanol content in relation to ultrapure water all had an impact on the UAE conditions. Utilizing UAE and UHPLC-DAD techniques, the developed method of ascorbic acid analysis is relatively environmentally friendly and selective. According to the current study, *C. medica* and *Z. spina-christi* fruits are important sources of ascorbic acid and polyphenols. The quadratic models used in this study were significant and well-fitted to the data for total phenolic content (TPC), EC50 values, and ascorbic acid. The models showed excellent correlations between experimental and predicted values. The signal-to-noise ratio and other fit statistics indicated the precision and significance of the models. The model terms were found to be important for calculating the different chemical parameters in the plant samples.

CRediT authorship contribution statement

Tilahun Belayneh Asfaw: Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Mesfin Getachew Tadesse:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Conceptualization. **Fekade Beshah Tessema:** Writing – review & editing, Software, Investigation, Data curation, Conceptualization. **Henock Woldemichael Woldemariam:** Writing – review & editing, Visualization, Validation, Supervision, Software, Data curation, Conceptualization. **Ajay V. Chinchkar:** Writing – review & editing, Visualization, Validation, Resources, Methodology. **Anurag Singh:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Conceptualization. **Ashutosh Upadhyay:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Conceptualization. **Bewketu Mehari:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Appendix

Table 1A
Two-way ANOVA for total phenolic, total flavonoid, and ascorbic acid contents of *C. medica* fruits, *C. medica* fruits peel, and *Z.spina-christi* fruit under different drying conditions extraction methods.

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	351720.6	8	43965.07	9.03501	2.16E-06	2.244396
Columns	1,262,558	4	315639.5	64.86527	6.8E-15	2.668437
Error	155714.5	32	4866.079			
Total	1,769,993	44				

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101310.

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