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Impact of Pre-treatment and Drying Temperature on Retention of Bioactives from Immature Dropped Kinnow Fruit using Ultrasonication

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ABSTRACT

Background: Agri-waste including immature dropped fruits is rich in various nutrients and bioactive compounds that can be dried and utilized for beneficial purposes.

Methods: Impact of pre-treatments (citric acid, steam blanching and both together) and drying temperatures (40-80°C) on retention of bioactive compounds from immature dropped kinnow fruit (IDKF) was studied using ultrasonication.

Result: Highest total phenolic (6.42 g GAE/100 g and 8.68 g TAE/100 g), total flavonoid (10.58 g QE/100 g), DPPH (390.79 mg AAE/100 g), FRAP activity (9.15 g TE/100 g and 4.65 g AAE/100 g) and minimum colour difference were detected in IDKF, pre-treated with citric acid and dried at 50°C. Crude IDKF bioactive extract, prepared from this sample showed antimicrobial activity against Gram positive bacteria. Impact of citric acid pre-treatment and drying temperatures on maximizing bioactive compounds retention was indicated by principal component analysis too. Total saponins and alkaloids were better retained in sample with no pretreatment and dried at 40°C.

Key words: Antimicrobial activity, Antioxidant activity, Bioactive compounds, Immature dropped kinnow fruit.

INTRODUCTION

Agri-waste, rich in valuable bioactive compounds (phenolics, flavonoids, anthocyanins, betaines and carotenoids), antioxidants, enzymes, dietary fiber, essential oils, pectin, etc. are of great interest for secondary purposes such as functional food, medicinal drugs, cosmetics industry, etc. Kinnow (*Citrus reticulata* Blanco), an important mandarin hybrid (*Citrus nobilis* × *C. deliciosa*) grown widely in North India, exhibits natural fruit drop, leading to favourable source-sink relationships for healthy growth of mature fruits. The drop of kinnow fruits is quite high, only 0.5-2% of bloom is turned to marketable fruits. Fresh droppings of fruits contain higher moisture (≥65%, w.b.) and susceptible to physical, chemical and microbial spoilage. Drying is one of the simplest and efficient methods that reduces the spoilage and enhanced the shelf-life but optimum drying conditions are important to prevent the negative impact of high or low temperature on overall quality of the dried products (Fulchand *et al.*, 2015). Treatments given prior to drying process have a significant impact on drying rate, quality of products and retention of antioxidants (Morais *et al.*, 2018).

The agriculture waste contained high amount of polyphenolic compounds, underwent serious deterioration due to enzymatic and non-enzymatic changes as the drying progress. Thermal blanching with hot water or steam is extensively used pre-treatment to inactivate the enzymes, responsible for quality degradation. In addition, citric acid pre-treatment is one of the most frequently used chemical for preventing browning as well improving the quality products (Shrestha *et al.*, 2020). Therefore, the present study was conducted to evaluate the impact of pre-treatments and drying temperatures on retention of bioactives of immature dropped kinnow fruit.

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MATERIALS AND METHODS

Sample collection, pre-treatments and drying

Immature green-coloured kinnow fruits (16.97±2.95 mm), collected in June, 2022 from a farmer's orchard in Abohar (Fazilka district, Punjab, India), were used for the study, which was conducted at ICAR-CIPHET, Ludhiana. The sliced (mean thickness 4.87±0.021 mm) samples were then subjected to various pre-treatments *viz.* control (untreated), citric acid (50mg/100g of sample), steam blanching (3 min) and combination of citric acid (50 mg/100 g) and steam blanching (3 min). Citric acid (CA) pre-treatment was given by spraying the solution (0.5%, 10 mL/100 g sample) with gentle mixing for about 30 seconds, blanching for 3 min and drying at 40, 50, 60, 70 and 80°C on wire mesh trays (800 mm × 400 mm × 30 mm) until ~5% moisture (w.b.) and stored at minus 4°C for quality analysis. The total drying time taken by the pre-treated samples was 8.5, 7, 6.5, 4.5 and 4 h, dried at 40, 50, 60, 70 and 80°C, respectively.

Bioactive compounds analysis

The crude extract was prepared from dried IDKF samples using 50% aqueous ethanol as solvent in ultrasonication bath at 45°C for 1 h. The extraction mixture (1:20 w/v sample and solvent) in amber colour glass beaker was kept in ultrasonicator, followed by centrifugation at 4000 rpm for 15 min and supernatant was collected and stored at 4°C for further analysis.

Total phenolics in samples (gallic acid, GAE/100 g and tannic acid equivalent, TAE/100g) was evaluated by Folin-Ciocalteu (FC) method as suggested by Arora *et al.* (2019; 2021). Total flavonoid content (TFC, quercetin equivalent, g QE/100 g) was quantified as per the method suggested by Rameshkumar *et al.* (2012). Antioxidant activity or DPPH Radical Scavenging Assay (ascorbic acid equivalent, mg AAE/100 g) was conducted following the standard procedure (Dudonné *et al.,* 2009; Mridula *et al.*, 2020). FRAP (Ferric Reducing Antioxidant Power) activity (Trolox/ ascorbic acid Equivalent (g TE/100 g and g AAE/ 100 g) was analyzed as per the method by Vergara-Salinas *et al.* (2012) and Aparna and Lekshmi, (2023). The total saponins (Diosgenin equivalent, mg DE/100 g) were measured using the process by Tan *et al.* (2014). Total alkaloid (Atropine equivalent, mg AE/100 g) was quantified with slight modification of method suggested by Shamsa *et al.* (2007).

Colour attributes

A hunter colorimeter model 45/0-L mini scan XE PLUS (Hunter Associates Labs, Reston, VA, USA) was used to assess colour values and colour difference (Chakrborty *et al.,* 2011 and Mridula *et al.,* 2017).

$$\text{Color difference, } \Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

Where,

ΔL = L-L0,

Δa = a-a0,

Δb = b-b0

L, a and b are values of pre-treated while L0, a0 and b0 are values of control dried samples.

Antibacterial activity

The crude extract of CA pre-treated sample (dried at 50°C) was evaluated for antibacterial activity against pathogens using agar well diffusion method (Campos *et al.,* 2022). Pure culture of Gram positive (*Bacillus cereus* and *Staphylococcus aureus*) and Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria (Department of Dairy Microbiology, GADVASU, Ludhiana) and Imipenem, standard antibiotic as positive control for bacterial inhibition were considered for the study. Zone of inhibition (ZOI) around the wells of IDKF crude extracts and respective controls were measured in millimeters (mm) using zone scale. The efficiency of IDKF crude extracts was measured by comparing their inhibition zone with imipenem. The value of AAI varies from -100% to 100%. AAI of -100% indicates that the extract showed no inhibitory activity. The values in range of -100% to 0% indicate that the extract had a lower inhibitory activity. AAI value of 0% signifies that the extract and control had same inhibition against test pathogen. AAI values ranging from 0 to 100% indicate that the extract had more inhibition halos than the control. AAI equal to 100% means the control showed no inhibition zones. The ZOI values were further used to determine the Antimicrobial Activity Index (AAI) using following equation (Vancheva *et al.,* 2015):.

$$\%\,\text{AAI} = -1 \times \frac{A - E}{A + E} \times 100$$

Where,

A = ZOI against Imipenem.

E = Represents the ZOI with IDKF crude extracts.

Statistical analysis

The data analysis was performed using SPSS for ANOVA and Duncan multiple range test. The Principal Component Analysis (PCA) was carried out using ‘FactoMineR’ and ‘factoextra’ packages in R software (Husson *et al.*, 2020; Kassambara and Mundt, 2020).

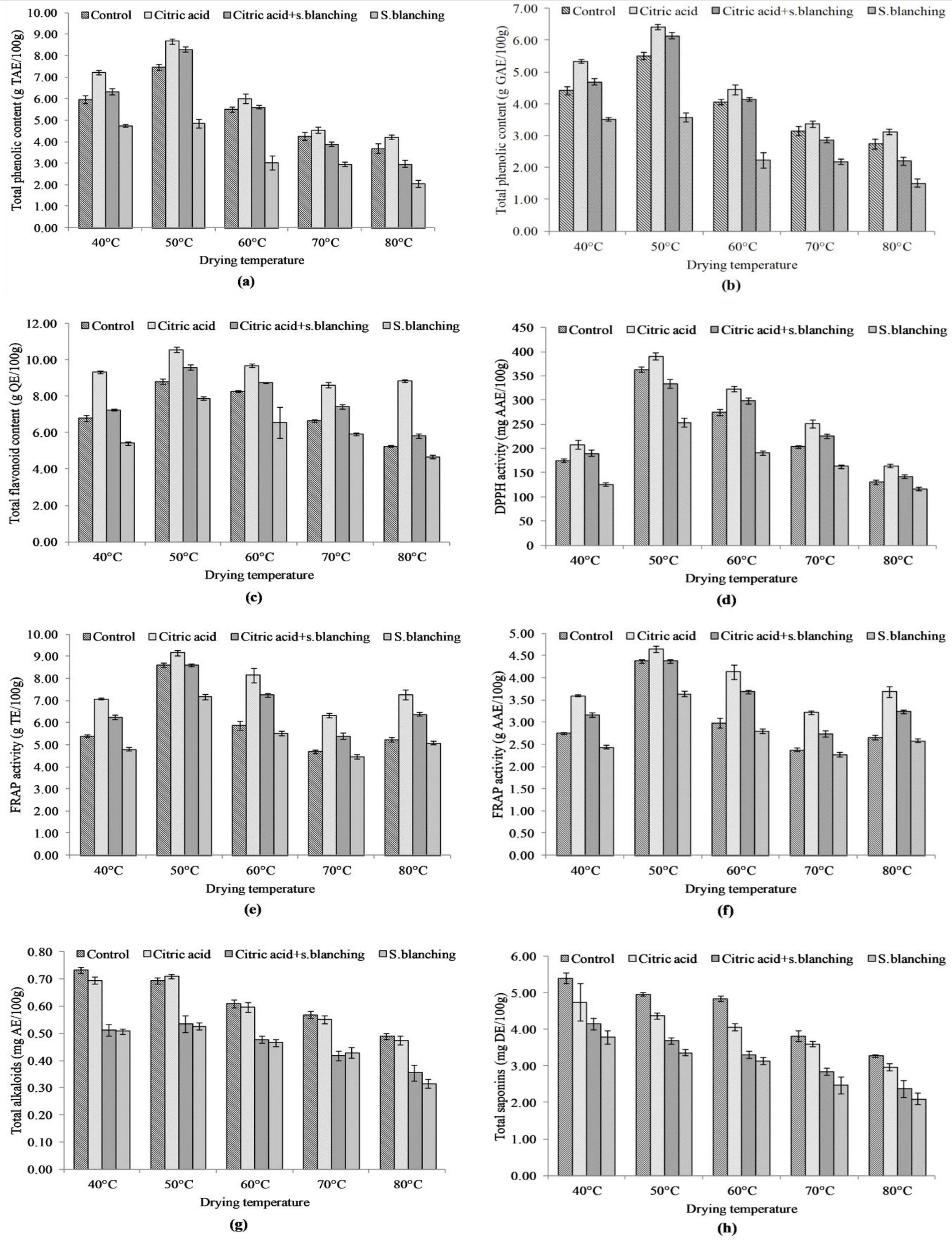
RESULTS AND DISCUSSION

Total phenolic content

Total phenolic content (TPC) of IDKF samples ranged from 1.519 to 6.423 g GAE/100 g and from 2.052 to 8.675 g TAE/ 100 g. Highest retention of TPC was observed in CA pre-treated IDKF samples dried at 50°C (Fig 1a and 1b), might be due to contribution of citric acid in delaying the oxidation of polyphenol components, by functioning as an anti-browning and chelating agent (Wang *et al.*, 2003) when used in combination with a lower drying air temperature. The percent retention of TPC in citric acid pre-treated IDKF samples dried at 50°C was more by 16.74, 30.73, 47.58 and 51.32% from samples dried at 40, 60, 70 and 80°C, respectively (Table 1). The significant ($p<0.05$) decline in the TPC as the drying temperature increased from 60 to 80°C, might be due to chemical degradation of phenolics (enzymatic reactions, maillard reactions and pigment degradation) at elevated temperature of drying. Drying at 40°C also resulted in loss of phenolic content, might be due to longer drying time and air exposure. Ahmed *et al.* (2010) also observed the negative impact of higher temperatures during thermal processing of sweet potato on phenolic compounds stability due to chemical disintegration.

Total flavonoid content

TFC of IDKF samples ranged from 4.681 to 10.576g QE/ 100 g (Fig 1c). Highest retention of TFC was observed in citric acid pre-treated IDKF samples dried at 50°C ($p<0.05$), might be due to contribution of CA in the reduction of the oxidation rate of flavonoids when combined with lower drying temperature. The retention of TFC in CA pre-treated IDKF samples dried at 50°C was more by 11.73, 8.39, 18.47 and 16.45% from samples dried at 40, 60, 70 and 80°C, respectively (Table 1), might be due to deterioration of



[(a)-Total phenolic content, g TAE/100 g; (b)-Total phenolic content, g GAE/100 g; (c)-Total flavonoid content, g QE/100 g; (d)-DPPH activity, mg AAE/100 g; (e)-FRAP activity, g TE/100 g; (f)-FRAP activity, g AAE/100 g; (g)-Total alkaloids, mg AE/100 g; (h)-total saponins, mg DE/100 g] in immature dropped kinnow fruit.

Fig 1: Effect of pre-treatment and drying temperatures on retention of functional compounds.

bioactive compounds, enzymatic reactions, maillard reactions and oxidation. Kolla *et al.* (2021) also reported lower flavonoids in doum fruit with increased temperature from 40 to 70°C.

Antioxidant activity (DPPH and FRAP)

DPPH activity of all the treated IDKF samples were ranged from 116.88 to 390.79 mg AAE/100 g (Fig 1d). Similar to phenolics and flavonoids, retention of DPPH activity was also higher in CA pre-treated IDKF samples, dried at 50°C thereafter drying at elevated temperature (from 60 to 80°C) resulted in loss of DPPH activity ($p<0.05$), could be due to degradation of polyphenols and flavonoids, which eventually resulted in decrease of antioxidant activity (Kuyu *et al.*, 2018). Retention of DPPH activity in CA pre-treated IDKF samples dried at 50°C was more by 46.74, 17.20, 35.67 and 57.89% from samples dried at 40, 60, 70 and 80°C, respectively (Table 1). Chan *et al.* (2009) also stated the reduction of antioxidant abilities of fruits and vegetables at higher temperatures, likely related to destruction of polyphenols that were earlier activated at low temperatures. FRAP activity of IDKF samples were ranged from 4.466 to 9.152g TE/100 g and 2.269 to 4.650 g AAE/100 g (Fig 1e, 1f) with maximum retention at 50°C. The percent retention of FRAP activity in citric acid pre-treated IDKF samples dried at 50°C was more by 22.67, 11.10, 30.73 and 20.73% from samples dried at 40, 60, 70 and 80°C, respectively (Table 1). Yen and Vu (2017) also stated retention of FRAP activity in *Limnophila aromatica* at 50°C comparable to drying at 40 and 60°C.

Total alkaloids and saponins

Alkaloids content in all the IDKF samples ranged from 0.31 to 0.73 m g AE/100 g ($p<0.05$) with higher retention in control samples followed by CA, CA and SB and SB. This decrease with increasing temperature from 40 to 80°C, might be due to destruction of alkaloids at elevated temperatures. The percent retention of total alkaloids in control IDKF samples dried at 40°C was more by 5.32, 16.78, 22.37 and 33.15% from samples dried at 50, 60, 70 and 80°C, respectively (Table 2). Zuiderveen *et al.* (2021) also reported decreased alkaloid content of goldenseal with increased drying temperature.

Saponins in IDKF samples (Fig 1h) ranged from 2.10 to 5.41 mg DE/100 g ($p<0.05$), with higher retention in control samples, followed by CA, CA+SB and SB and decreased with increasing drying temperature from 40 to 80°C. Although the drying time was reduced by elevated drying temperature, but retention of saponin components was found more at lower drying temperature. The percent retention of total saponins in control IDKF samples dried at 40°C was more by 8.04, 10.23, 29.25 and 39.39% from samples dried at 50, 60, 70 and 80°C, respectively (Table 2). Ning *et al.* (2015) also reported to dry at low temperature for higher saponins in *red ginseng*.

Colour quality of IDKF

The IDKF samples dried after steam blanching and combined treatments were found to have highest L, a and b values. The 'L values' of IDKF ranged from 54.78±0.43 to 60.68±0.26, 55.63±0.21 to 59.43±0.13, 56.55±0.13 to

Table 1: Enhanced TPC, TFC and antioxidant activity in IDKF samples dried at 50°C.

Drying temperatures, °C	Enhanced by % at 50°C			
	TPC	TFC	% DPPH inhibition	FRAP activity
		Control		
40	19.95	22.65	51.70	37.15
60	26.24	6.16	24.46	31.76
70	42.68	24.30	44.00	45.54
80	50.25	40.29	63.98	39.19
		Citric acid		
40	16.74	11.73	46.74	22.67
60	30.73	8.39	17.20	11.10
70	47.58	18.47	35.67	30.73
80	51.32	16.45	57.89	20.73
		Steam blanching		
40	2.01	31.17	50.26	32.95
60	37.68	17.01	24.59	22.97
70	38.74	24.89	35.63	37.65
80	57.70	40.67	53.98	28.96
		Citric acid + steam blanching		
40	23.48	24.24	43.00	27.50
60	32.34	8.68	10.45	15.75
70	53.22	22.44	32.51	37.27
80	64.06	39.29	57.23	25.79

64.35±0.17 and 57.48±0.71 to 65.03±0.10 in control, CA, CA + SB and SB samples, respectively at different drying temperature. The 'a values' ranged from 0.55±0.13 to 1.00±0.08, 0.56±0.05 to 1.03±0.10, 0.71±0.01 to 2.38±0.13 and 0.83±0.15 to 2.60±0.08 in control, CA, CA + SB and SB samples, respectively at different drying temperature. The 'b values' ranged from 18.13±0.21 to 21.08±0.29, 18.68±0.22 to 20.63±0.15, 18.33±0.42 to 22.35±0.53 and 19.35±0.59 to 22.30±0.22 in control, CA, CA + SB and SB samples, respectively at different drying temperature. Colour difference (ΔE) between control and pre-treated samples dried at different temperature, was higher in steam blanched samples dried at 60°C whereas minimum was observed in citric acid pre-treated sample dried at 50°C. This might be due to prevention of enzymatic browning by citric acid treatment. Similar results were reported by Brar *et al.* (2020) for Yellow European Plums drying at elevated temperature.

Principal component analysis (PCA)

PCA was used to determine the impact of primary components across a variety of treatment combinations viz., pre-treatment and drying temperature on bioactive compounds and colour quality of IDKF. Total of eleven principal components (PCs) were collected that revealed three most informative PCs with Eigen values of 6.58, 2.67 and 0.87, which accounted for 92.14% of the cumulative variance. The PC's having Eigen values of more than 1 are considered as most informative PC's. The first principal component (Dim 1) had the highest eigen value of 6.58, explained 59.84% of statistical variance while second principal component (Dim2) had eigen value of 2.67, explained 24.3% of statistical variance (Fig 2a).

A small angle suggested a positive correlation while large angle shows a negative correlation. However, the angle 90° indicates no correlation between the given treatments. Based on the statistical analysis of loading plot (Fig 2b), it was observed that TP.TA, TP.GA, TFC, DPPH, FR.TE, FR.AA, alkaloids and saponins form narrow angles (less than 90°) and are located in the same quadrant depicts the strong correlation and affects each other directly. The PCA biplot (Fig 2c) depicted sample differences in terms of extraction of bioactive components. The PCA showed that independent variables *viz*. PT2 (Control, 50°C), PT3 (Control, 60°C), PT6 (Citric acid (CA), 40°C), PT7 (CA, 50°C), PT8 (CA, 60°C) and PT12 (CA+SB, 50°C), lies in same quadrant on the upper and lower positive side on the Dim1 axis (Fig 2c) were influential in maximizing the retention of bioactives from IDKF samples with studied processing conditions. PCA results indicated the impact ($p<0.05$) of pre-treatments and drying temperatures on retention of bioactive compounds from IDKF samples and selection of few points for maximum retention of bioactive compounds.

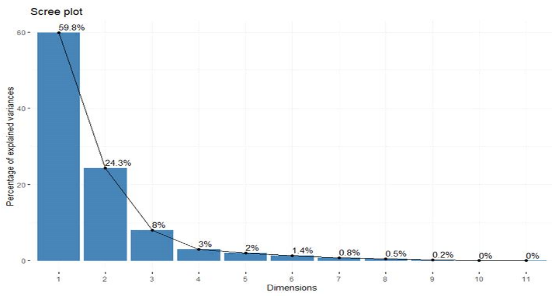
Table 2: Enhanced (by percentage) total alkaloids and saponins in IDKF samples dried at 40°C.

Drying temperatures, °C	Enhanced by % at 40°C	
	Total alkaloids	Total saponins
	Control	
50	5.32	8.04
60	16.78	10.23
70	22.37	29.25
80	33.15	39.39
	Citric acid	
50	-2.01	7.88
60	14.37	14.32
70	20.69	24.11
80	31.90	37.71
	Steam blanching	
50	-3.74	11.23
60	8.27	17.08
70	15.55	34.81
80	37.80	44.56
	Citric acid + steam blanching	
50	-4.49	11.08
60	6.64	20.24
70	18.16	31.25
80	30.47	42.69

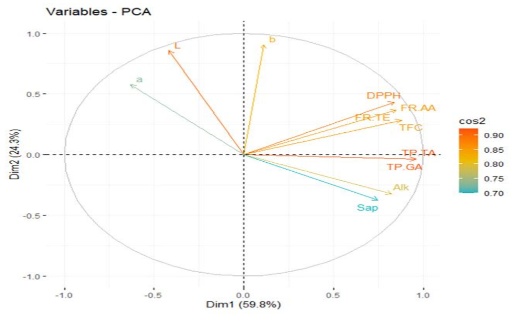
Antibacterial activity

This study showed that Gram negative bacterial test strains (*E. coli* and *Pseudomonas*) showed marked resistance to IDKF crude extract up to 25 mg/mL concentration (Table 3). Moreover, Gram positive bacterial strain *i.e. Bacillus* (14±1) and *Staphylococcus* (15±1) showed inhibition zone at the concentration of 25 mg/mL. It was observed that Gram positive strains (*S. aureus* and *B. cereus*) were more sensitive to IDKF crude extracts as compared to Gram negative strains. The variation in sensitivity among bacterial strains could be due to cell envelope structure, which hinders the penetration of antimicrobial substances, thus providing more resistance to Gram-negative bacteria (Pavlić *et al.,* 2017). Antimicrobial potential of IDKF crude extract is in the line with the literature related to kinnow peel and pomegranate peel extract. Safdar *et al.* (2017) reported *S. aureus* to be more sensitive towards kinnow peel extracts. The results of the present study are in line to the earlier findings by Campos *et al.* (2022) and Hanani *et al.* (2019) who reported the sensitivity of *S. aureus* (up to 14 mm of inhibition zone) against pomegranate extracts and confirmed the results of present study with zone of inhibition (15 mm) against the *Staphylococcus*.

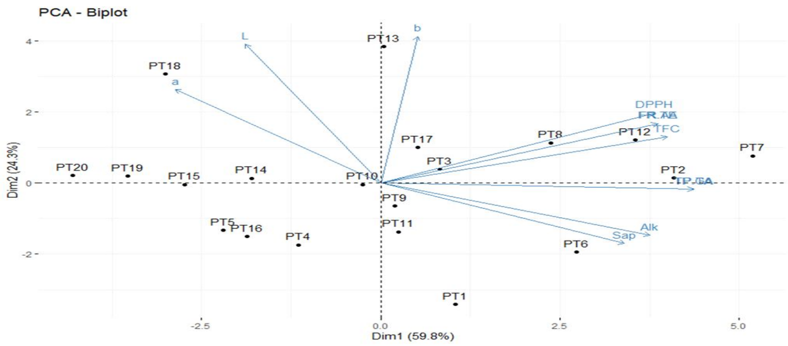
The antimicrobial activity index (AAI) of IDKF crude extract, against Imipenem was found negative (Table 3) indicating higher inhibitory activity of Imipenem against bacteria than the IDKF crude extract. Imipenem is a broad spectrum carbapenem antibiotic and has antibacterial activity against Gram positive and Gram-negative bacteria. AAI values at 25 mg/mL for *Bacillus* and *Staphylococcus*



2a. Scree plot



2(b) Loading plot



2(c) Biplot

(a) Scree plot; (b) Loading plot (c) Biplot {PT1: Control, 40°C; PT2: Control, 50°C; PT3: Control, 60°C; PT4: Control, 70°C; PT5: Control, 80°C; PT6: Citric acid (CA), 40°C; PT7: CA, 50°C; PT8: CA, 60°C; PT9: CA, 70°C; PT10: CA, 80°C; PT11: CA+Steam blanching (SB), 40°C; PT12: CA+SB, 50°C; PT13: CA+SB, 60°C; PT14: CA+SB, 70°C; T15: CA+SB, 80°C; PT16: SB, 40°C; PT17: SB, 50°C; PT18: SB, 60°C; PT19: SB, 70°C; PT20: SB, 80°C; TP.TA: Total phenolic content in terms of tannic acid equivalent; TP.GA: Total phenolic content in terms of gallic acid equivalent; TFC: Total flavonoid content; FR.TE: FRAP activity in terms of Trolox equivalent; FR.AA: FRAP activity in terms of ascorbic acid equivalent; Alk: total alkaloids; Sap: Total saponins}.

Fig 2: Principal component analysis on effect of pre-treatments and drying temperature on functional compounds and colour parameters of immature dropped kinnow fruit.

Table 3: Zone of inhibition and antimicrobial activity index of IDKF extract against the selected micro-organism.

IDKF extract (concentration)	Zone of inhibition (mm)			
	E. coli	*Pseudomonas*	*Bacillus*	*Staphylococcus*
25 mg/ml	R	R	14±1	15±1
10 mg/ml	R	R	R	R
5 mg/ml	R	R	R	R
2.5 mg/ml	R	R	R	R
1.25 mg/ml	R	R	R	R
0.63 mg/ml	R	R	R	R
0.31 mg/ml	R	R	R	R
Antimicrobial activity index (%) against imipenem				
25 mg/ml	-100	-100	-12.5±1	-6.25±1
10 mg/ml	-100	-100	-100	-100
5 mg/ml	-100	-100	-100	-100
2.5 mg/ml	-100	-100	-100	-100
1.25 mg/ml	-100	-100	-100	-100
0.63 mg/ml	-100	-100	-100	-100
0.31 mg/ml	-100	-100	-100	-100

R: Resistant.

were closer to 0%, which indicated that inhibition zone of IDKF crude extract is closer to Imipenem's inhibition zone. However, AAI value at all the studied level of IDKF crude extract for *E. coli* and *Pseudomonas* had values of -100% that showed that none of the studied extract concentration inhibited this Gram-negative bacterium. The results of the present study suggested that crude extract of IDKF possess the antimicrobial activity against Gram positive bacteria.

CONCLUSION

The pre-treatments and drying temperature had significant effect on bioactive compounds and colour quality of immature dropped kinnow fruit. Citric acid (50 mg/100 g) pre-treatment and drying at 50°C temperature was found the most effective treatment in maximum retention of TPC, TFC and antioxidant activity (DPPH and FRAP) of dried IDKF samples as compared to other conditions. The saponins and alkaloids were better retained in control sample, dried at 40°C. The citric acid pre-treatment prior to drying also improves the colour attributes of dried IDKF samples. The crude bioactive extract from IDKF, prepared from sample with citric acid pre-treatment and dried at 50°C showed the antimicrobial activity against Gram positive bacteria, indicating the applicability of this pre-treatment and drying temperature to maximize the retention of bioactives in IDKF for its further utilization for beneficial purposes.

These findings highlight the potential of IDKF as a low-cost, sustainable source of bioactive compounds with antioxidant and antimicrobial properties. Such processed IDKF material-either in the form of dried powder or concentrated extract-can be developed into value-added products for use in functional foods, nutraceuticals, herbal supplements, or even natural cosmetic formulations. This could create new opportunities for the commercial utilization of agri-waste while contributing to waste valorization and circular economy initiatives in the food and wellness industries.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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