

# Influence of Extraction Techniques on Betalain Yield and Bioactive Phytochemical Analysis of Nopal Fruit Peels

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## ABSTRACT

**Background:** Nopal fruits (*Opuntia ficus-indica*) and their peels contain significant amount of natural colorants which can be used in food and pharmaceutical applications instead of using synthetic dyes. **Materials and Methods:** The present study focuses on the extraction of betalain from nopal fruit peel by conventional and Ultrasound-Assisted Extraction (UAE) and optimizing the UAE process variables using Box Box-Behnken surface design, as well as a preliminary screening of valuable phytochemicals using Gas-Chromatography and Mass Spectroscopy (GC-MS) which contribute to the medicinal potential of nopal fruits. **Results:** Soxhlet extraction of Betacyanin and Betaxanthin (BC and BX) was found to be 4.51 mg/g of BC and 0.67 mg/g of BX. The extraction yield of betacyanin and betaxanthin for UAE was extracted at 16 min, 40°C, 1/22 g/mL, and the yield was found to be  $3.00 \pm 0.23$  mg/g BC,  $0.225 \pm 0.02$  mg/g BX. **Conclusion:** GC-MS analysis of nopal fruit (*Opuntia ficus-indica*) peel revealed that ethanol extracts of nopal fruit peel revealed two fold higher results of - (+)-Ascorbic acid 2, 6-dihexadecanoate of UAE than soxhlet. Also other phytochemicals such as n-Hexadecanoic acid (Palmitic acid) which possesses anti-inflammatory, antibacterial and antifungal activity are found to be higher.

**Keywords:** Natural colorant, Ultrasound-assisted extraction, Response surface methodology, Anti-inflammatory, Anti-bacterial.

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## INTRODUCTION

Currently, Pigment research is gaining popularity, as colour is considered as a predominant factor among many characteristics of food.<sup>1</sup> Most food colorants are derived from synthetic processes. Regular consumption of these artificial colorants can create several health effects on human beings such as sleep disorders and irritability.<sup>2</sup> Since there are risks of artificial colors to human health extracting colorants from natural sources is the only alternative source for replacing this synthetic colours.<sup>2</sup> There are four main categories of natural pigments (chlorophylls, carotenoids, betalain and anthocyanins) which are abundant in nature.<sup>3</sup> Nopal (*Opuntia ficus-indica*) fruits an edible cactus plant, also known as Prickly pear, Indian fig opuntia, or Cactus pear is widely found all over India. This plant has sharp leaves with spines. Colourful flowers grow on these leaves that develop into fruits. The fruit extract possesses various pharmacological properties and also substantial amounts of eicosanoic acid which possesses antioxidant property, hexadecanoic acid, ethyl ester which

possess Anti-bacterial and anti-fungal activity, Nevertheless it also possesses strong colour viz betalain as natural pigment. leading to the development of natural colorants to replacing synthetic ones. So, Pigment Extraction is one of the beneficial approaches and an alternative to replace synthetic colorants. The amalgamation of purple-colored betacyanin and the yellow-colored betaxanthin constitutes the colour of nopal fruits (*Opuntia ficus-indica*) named Betalain.<sup>4,5</sup> Normally conventional methods: Soxhlet extraction, Maceration, Solvent assisted extraction has been used for extraction. In order to overcome the disadvantages (longer extraction time, efficiency and higher solvent consumption) advanced extraction techniques have been considered as an alternative to conventional methods.<sup>6</sup> Ultrasound extraction is proposed as one of the feasible techniques in modern extraction<sup>7</sup> for the extraction of chlorophylls.<sup>8</sup> Since, ultrasound waves enhances the extraction efficiency<sup>9</sup> due to the disruption of cell walls.<sup>10</sup> It also enables greater yield in minimum time and a significant reduction in solvent consumption.<sup>11</sup> In the present study, ultrasound-assisted extraction was used for natural colorant extraction from nopal fruit (*Opuntia ficus-indica*) peel by optimizing the process variables using Response Surface Methodology (RSM). The presence of phytochemicals and yield of extract obtained from UAE was compared with the conventional soxhlet method and the structural modifications were also studied.



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## MATERIALS AND METHODS

### Sample Preparation

Nopal fruits (*Opuntia ficus-indica*) were collected from a village called Nallampalli near Dharmapuri district, Tamil Nadu, India. The fruits were cleaned, peeled and shade-dried at room temperature. It is then completely dried in an oven (Sigma Scientific Instruments, India) to remove moisture content and ground. The fine powder is packed tightly and stored.

### Soxhlet extraction

Soxhlet extraction is a common and a very efficient method used for the extraction of valuable organic compounds from solid materials. The repeated extraction cycles ensures that all of the compounds are extracted. The solid material to be extracted is placed in a thimble which is placed inside a glass extraction chamber. Solvent (ethanol) is added to the extraction chamber. The chamber is then heated, causing the solvent to boil and evaporate. The evaporated solvent rises to the top of the chamber and condenses. This condensed solvent then drips back down into the extraction chamber. The dripping solvent gradually extracts the organic compounds from the solid material inside the thimble. The extraction process continues until the solvent in the extraction chamber becomes saturated with the extracted compounds.

### Ultrasound-Assisted Extraction (UAE)

The extraction of natural colorant was carried out using an Ultrasonic (bath) device (LABMAN, Chennai, India) with a constant power supply of 24 kHz. Three extraction variables were taken and their maximum and minimum levels are as follows: Ethanol concentration of 15 to 25 mL/g, extraction temperature of 30 to 45°C and time duration of 5 to 25 min.

### Betacyanin (BC) and Betaxanthin Content (BX) determination

Betaxanthin and Betacyanin concentration is determined using UV spectrophotometer at 535 nm and 480 nm, individually<sup>12,13</sup> and was calculated using the following standard equation:

$$BC / BX [mg/g] = \frac{A (DF) (MW) V_d}{\epsilon L W_d} \quad (1)$$

Where

A = absorption value (maximum absorption of 535 nm).

DF = dilution factor,

$V_d$  = volume of peel solution (mL),

$W_d$  = dry weight of peel (g) and,

L = path-length (1 cm) of the cuvette.

The molar elimination coefficients (e) (MW=550 g/mol; e=60,000 L/mol cm in H<sub>2</sub>O) and (MW=308 g/mol; e=48,000 L/mol cm in H<sub>2</sub>O) were used.

### Morphological Analysis (SEM Analysis)

The morphological changes of the solid matrix were analyzed using a Scanning Electron Microscope (SEM) (a Tescan Vega3-SBU (Czech Republic country). In order to identify the physical changes in the solid matrix due to ultrasound, the solid matrix was subjected to analysis before and after the extraction process.

### Phytochemical Analysis (GC-MS Analysis)

The phytochemicals of crude extracts obtained from Soxhlet and UAE methods were analyzed using the GC-MS technique. The chemical constituents of the extracts were determined using Bruker 45X- GC-44 (GC) and SC-ION (MS) equipped with a 15 m interface fused silica capillary column with helium as carrier gas. The temperature in the oven was maintained at 45°C initially, and was raised to 70°C with a holding time of 5 min, and then the temperature was increased to 280°C with a heating rate of 8°C/min, 10 min maintained as holding time. The flow rate conditions are as follows; injection volume of 1 µL and a flow rate of the sample at 1 mL/min.

### Box-Behnken Design (BBD)

Box-Behnken Design (BBD) was employed for the optimization of UAE process variables to obtain a maximum extraction yield. In addition, the individual and interaction effects of variables on the colorant yield were also studied using 3D surface plots developed by BBD design. The relationship between the experimental factors involved and their corresponding responses was analyzed using a statistical model. The levels (minimum and maximum) of each factor involved in the process were determined using the following equation:<sup>14-16</sup>

$$X_i = \frac{X_{i1} - X_{i2}}{\Delta X_i} \quad \text{Where } i = 1, 2, 3 \dots k \quad (2)$$

Based on the factors involved in the process, the required number of experiments with different combinations was calculated using given equation.

To calculate the number of experiments (N) the following equation was used,

$$N = 2N(k - 1)C_o \quad (3)$$

Where k is the number of factors and  $C_o$  is the number of central points.

## RESULTS

The experimental and ANOVA results for ultrasound-assisted extraction were shown in Tables 1 and 2 and also comparison of both the methods were shown in Table 3. Figures 1 and 2 shows the effect of UAE process variables on BC and BX: a) F/S ratio vs. Temperature b) F/S ratio vs. Time c) Temperature vs. Time. Whereas, Figure 3 shows the SEM image results of Nopal fruit (*Opuntia ficus-indica*) peel for raw material, soxhlet extract and UAE for ethanol. While Figure 4a and 4b shows the results of GC-MS chromatogram for soxhlet extraction and UAE of nopal fruit peel.

### Fitting of second order polynomial equation and statistical analysis

The relationship between dependent and independent variables was analyzed using a multiple regression model. Based on the experimental value, the developed second-order polynomial equation of responses is given below:

$$BC = +2.97 + 0.25 X_1 + 0.25 X_2 + 0.091 X_3 - 0.18 X_1 X_2 + 0.26 X_1 X_3 - 0.43 X_2 X_3 - 0.60 X_1^2 - 0.58 X_2^2 - 1.08 X_3^2 \quad (4)$$

$$BX = +0.23 - 0.039 X_1 + 0.014 X_2 + 0.015 X_3 - 0.005 X_1 X_2 - 0.0075 X_1 X_3 - 0.027 X_2 X_3 - 0.012 X_1^2 - 0.077 X_2^2 - 0.085 X_3^2 \quad (5)$$

Where,

$X_1$  - Feed solvent ratio (g/mL)

$X_2$  - Temperature ( $^{\circ}\text{C}$ ),

$X_3$  - Time (min),

Analysis of Variance (ANOVA) was used to understand the fitness and suitability of the developed models and their results are shown in Table 2. It can be seen that both model responses have  $p$ -value  $< 0.0001$ , which indicates the consistency and accuracy between the experimental and predicted values<sup>17,18</sup>  $R^2$  adj values (0.9995 for BC and 0.9807 for BX) were found to be equal to  $R^2$  (0.9998 for BC and 0.9916 for BX), results revealed that the models can be explained by more than 98% value. Therefore, the developed models could be used to investigate the extraction process carried out in this study. The "Lack of fit"  $p$ -value of predicted models (0.0568 and 0.9136 for BC and BX, respectively) is greater than (0.05), which implies that developed models equations were sufficient to determine the extraction yield.<sup>19</sup>

## DISCUSSION

### Soxhlet Extraction of Nopal fruit (*Opuntia ficus-indica*) Peel

For Soxhlet extraction, the experiments were carried out with a constant feed solvent ratio of 10:250 g/mL, a temperature of  $50^{\circ}\text{C}$ , and various time intervals from 2 to 8 hr. The higher

colorant results betacyanin content (4.51 mg/g) and betaxanthin content (0.67 (mg/g) were obtained at 6 hr. Initially, there was a significant increase in betacyanin and betaxanthin content which may be due to the rapid dissolution of solute on solid matrix and the higher mass transfer rate of the fresh solvent.<sup>20</sup> There were no significant change as the time is further increased, from 6 hr to 8 hr. This could be due to the possibility of denaturing of phenolic compounds, when time is increased beyond a certain limit.<sup>21</sup> Soxhlet extraction gives marginally higher yield than UAE due to maximum solvent consumption and longer extraction time of the soxhlet extractor resulting in increasing polyphenols yield.

### Effect of UAE process variables on BC and BX in nopal fruit (*Opuntia ficus-indica*) peel extract

#### Effect of time on BC and BX in nopal fruit (*Opuntia ficus-indica*) peel extract

One of the most prominent process parameter influencing the extraction of BC and BX is extraction time. Figure 1 shows that the BC and BX of nopal fruit extracts increased gradually from 0.52 mg/g BC to 2.98 mg/g BC and 0.01 mg/g BX to 0.21 mg/g BX when extraction time is increased from 15 min to 20 min. The rate of mass transfer is high during the initial stages of extraction, leading to higher yield. After 25 min, the colorants began to stabilize sharply as a final equilibrium might be reached leading to changes in colorant yield.<sup>22</sup> Further increasing the time might increase the chance of degradation of compounds.<sup>21</sup>

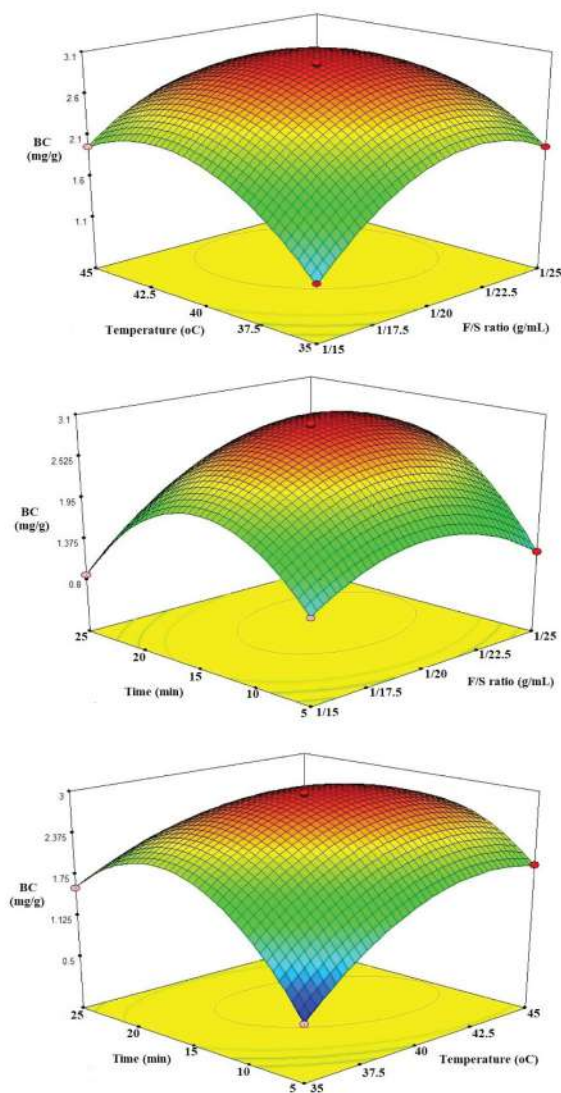
#### Effect of temperature on BC and BX in nopal fruit (*Opuntia ficus-indica*) peel extract

The extraction temperature is equally important as that of extraction time. Results indicated that, a significant increase of BC from 1.13 to 2.98 mg/g and 0.09 to 0.24 mg/g of BX was observed with an increasing temperature. This may affect the solvent properties.<sup>23,24</sup> as the viscosity of the solvent reduce and the solvent penetrates deep into the sample.<sup>25</sup> in higher extraction efficiency.<sup>17,26</sup> Pigment diffuses rapidly at higher temperature due to the molecular movement. From Figure 2 it is noted that by increasing the temperature from  $35$  to  $40^{\circ}\text{C}$ , total betaxanthin content is increased significantly. Nevertheless, above  $40^{\circ}\text{C}$ , the extraction yield significantly decreases. The reason could be either due to the possibility of solvent vaporization<sup>23</sup> or degradation of heat-sensitive natural colorant components.

#### Effect of feed-solvent ratio on BC and BX in nopal fruit (*Opuntia ficus-indica*) peel extract

An increase in solvent volume can increase the diffusion rate, absorption rate and swelling rate of cell wall (raw material). The maximum (Figures 1 and 2) yield of betalain was obtained at 1:20 g/mL. As the feed-solvent ratio was elevated from 1/15 to 1/20, increase in phenolic compounds from 1.97 to 2.96 mg/g for BC and 0.16 to 0.23 mg/g for BX was noticed. This is due to the reason



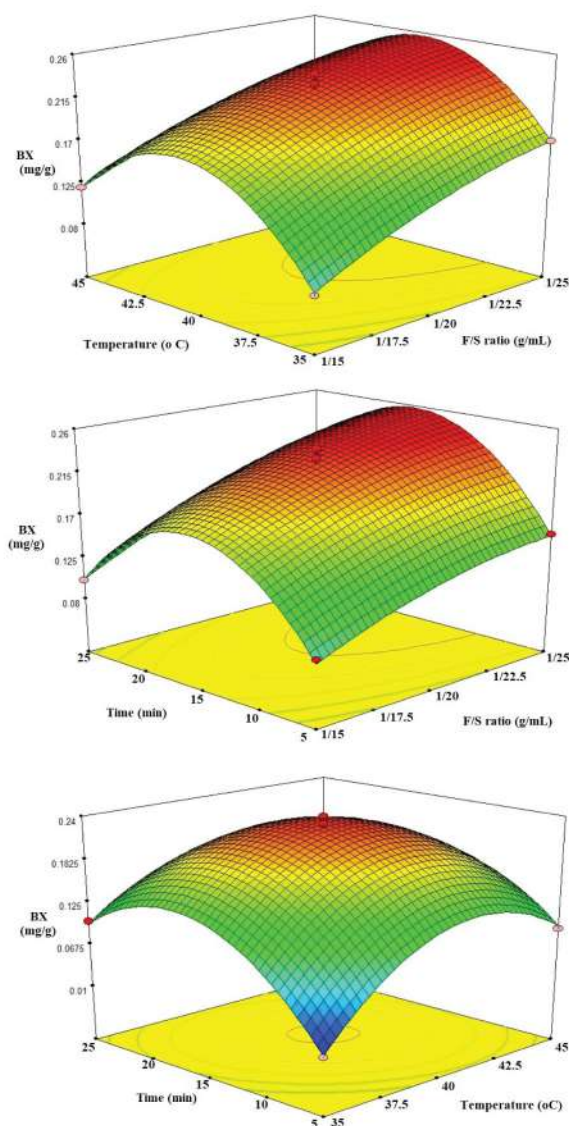


**Figure 1:** Effect of UAE process variables on BC: a) F/S ratio vs. Temperature b) F/S ratio vs. Time c) Temperature vs. Time.

that a large volume of solvent can dissolve targeted compounds more effectively. When F/S ratio is further increased to 1:25 g/mL, the betalain yield is found to decrease gradually (1.20 mg/g of BC and 0.15 mg/g of BX) as the F/S ratio attained a certain range, and the extract may be starting to degraded.<sup>27</sup> The experimental values are given in Table 1.

### Optimization and validation

Based on the developed regression model analysis, the numerical optimum condition for the maximum natural colorant yield was: feed solvent ratio of 1/21.5 g/mL, temperature of 39.6°C, time of 16.6 min and BC yield of 3.16 mg/g, BX yield of 0.229. According to the practical application, the conditions were slightly modified as: feed solvent ratio of 1/22 g/mL, temperature of 40°C, time of 17 min and the experiments were triplicated with an average

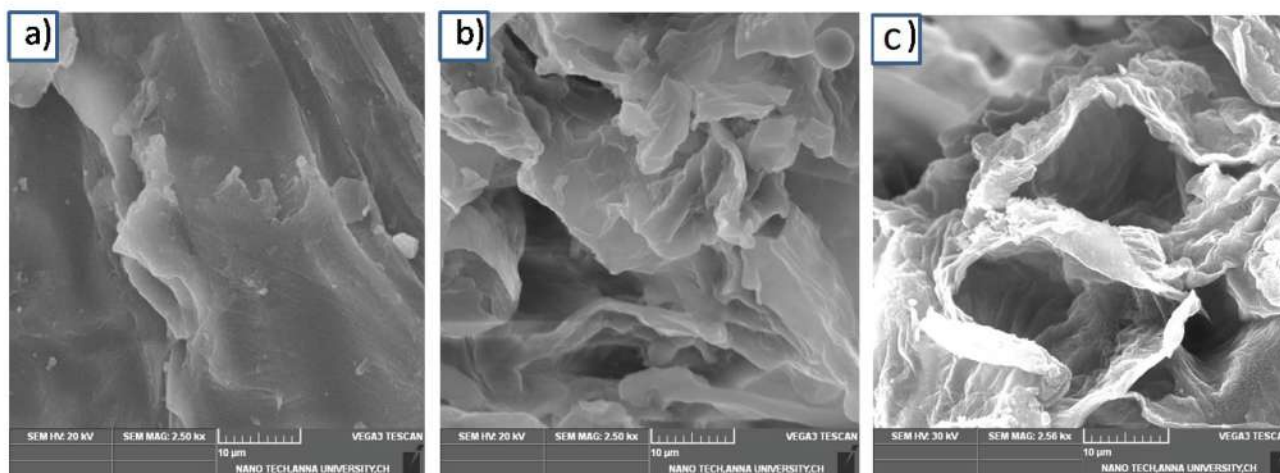


**Figure 2:** Effect of UAE process variables on BX: a) F/S ratio vs. Temperature b) F/S ratio vs. Time c) Temperature vs. Time.

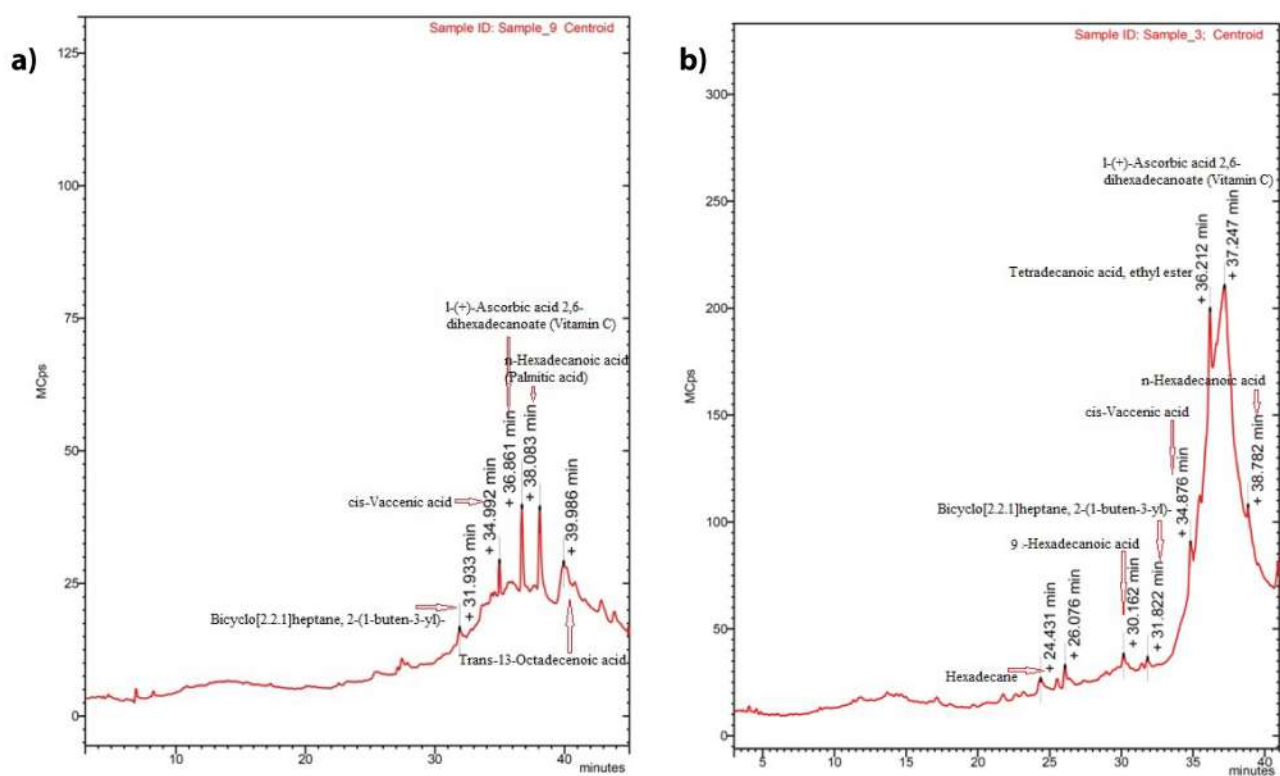
value of  $3.00 \pm 0.23$  mg/g BC,  $0.225 \pm 0.02$  mg/g BX respectively. It can be seen that the average value as well in agreement with software developed values.

### Comparison of conventional methods with ultrasound-assisted extraction

Soxhlet extraction of BC and BX from nopal fruit (*Opuntia ficus-indica*) peel was found to yield the largest extractable content of BC of 4.51 mg/g and BX of 0.67 mg/g. UAE possessed similar extraction yield as soxhlet extraction. The maximum extraction yield from nopal fruit (*Opuntia ficus-indica*) peel was found to be 3.00 mg/g BC and 0.225 mg/g BX. From the results it is noted that soxhlet extraction gives marginally higher yield than UAE which can be attributed to the fact that increase in yield is due to elevated temperature and time. Nevertheless, higher temperatures and



**Figure 3:** SEM image of Nopal fruit peel a) raw material b) soxhlet extract c) UAE for ethanol.



**Figure 4: a and b:** GC-MS chromatogram for soxhlet extraction and ultrasound-assisted extraction of nopal fruit peel.

longer duration may also degrade certain bioactive compounds as well.<sup>28</sup> Figure 3a shows the SEM image of raw material with a smooth surface before extraction process. Figure 3b illustrated the SEM image after soxhlet extraction conducted for 6 hr. The surface is mildly ruptured so that the solute passes through and probability of penetration of solvent into the solid surface is less.<sup>29</sup> Figure 3c depicts that SEM image of ultrasound-assisted extract where the rupture is higher comparatively due to ultrasonic cavitation caused significant structural changes on the surface by

producing a large number of cracks and increasing the efficiency of mass transfer by promoting diffusion.<sup>30</sup>

### Identification of phytochemical compounds

The qualitative GC-MS analysis in Table 4 revealed the presence of valuable phytochemicals of ethanol extracts from nopal fruit (*Opuntia ficus-indica*) peel. The component - (+)-Ascorbic acid 2, 6-dihexadecanoate which possess anticancer activity was identified to be 4 fold higher in extracts of UAE (69.95%) compared to soxhlet (17.86%). Compounds like n-hexadecanoic

**Table 1: Experimental result for Ultrasound-Assisted Extraction (UAE).**

Sl. No.	F/S (X1) (g/mL)	Temp (X2) (°C)	Time (X3) (min)	Ultrasound-assisted extraction	
				BC (mg/g)	BX (mg/g)
1	-1 (1/15)	1 (45)	0 (15)	1.97	0.12
2	-1 (1/15)	0 (40)	1 (25)	0.86	0.10
3	-1 (1/15)	-1 (35)	0 (15)	1.13	0.08
4	0 (1/20)	0 (40)	0 (15)	2.96	0.23
5	-1 (1/25)	0 (40)	-1 (5)	1.20	0.09
6	0 (1/20)	-1 (35)	1 (25)	1.56	0.10
7	0 (1/20)	0 (40)	0 (15)	2.98	0.24
8	0 (1/20)	0 (40)	0 (15)	2.96	0.24
9	0 (1/20)	1 (45)	-1 (5)	1.91	0.09
10	1 (1/25)	-1 (35)	0 (15)	1.97	0.17
11	0 (1/20)	1 (45)	1 (25)	1.23	0.07
12	0 (1/20)	-1(35)	-1 (5)	0.52	0.01
13	1 (1/25)	0 (40)	1 (25)	1.91	0.19
14	0 (1/20)	0 (40)	0 (15)	2.97	0.23
15	0 (1/20)	0 (40)	0 (15)	2.98	0.21
16	1 (1/25)	0 (40)	-1 (5)	1.20	0.15
17	1 (1/25)	1 (45)	0 (15)	2.10	0.19

**Table 2: ANOVA results for Ultrasound assisted extraction.**

UAE	Betacyanin (BC)		Betaxanthin (BX)	
Source	p-value	Coefficient Estimate	p-value	Coefficient Estimate
Model	< 0.0001	2.97	< 0.0001	0.23
X1 - F/S ratio	< 0.0001	0.2525	< 0.0001	0.03875
X2 - Temp	< 0.0001	0.25375	0.0055	0.01375
X3 - Time	< 0.0001	0.09125	0.0035	0.015
X1 X2	< 0.0001	-0.1775	0.3424	-0.005
X1 X3	< 0.0001	0.2625	0.1705	0.0075
X2 X3	< 0.0001	-0.43	0.0008	-0.0275
X12	< 0.0001	-0.595	0.0348	-0.0125
X22	< 0.0001	-0.5825	< 0.0001	-0.0775
X32	< 0.0001	-1.0825	< 0.0001	-0.085
Lack of Fit	0.0568		0.9136	
R <sup>2</sup>	0.9998		0.9916	
Adj R <sup>2</sup>	0.9995		0.9807	
Pred R <sup>2</sup>	0.9973		0.9733	
Adeq Precision	178.44		29.045	

**Table 3: Comparison of the conventional method with Ultrasound-assisted extraction.**

Extraction methods	Process Variables			Betain	
	Time(min/h)	Temp(°C)	F/S(g/mL)	BC(mg/g)	BX(mg/g)
Soxhlet extraction	6 hr	50	10/250	4.51	0.67
UAE	16 min	40	1/22	3.00	0.225

**Table 4: Constituents of ethanol extracts of nopal fruit peel and their relative percentages of total chromatogram area.**

Sl. No.	Bioactive Compounds	Ethanol		Pharmacological activity
		Soxhlet extraction	UAE	
1	1-(+)-Ascorbic acid 2,6-dihexadecanoate.	17.86	69.95	Anti-cancer, antioxidant.
2	Trans-13-Octadecenoic acid.	0.381	0.977	Cancer preventive, hypocholesterolemic, Anti-inflammatory. <sup>32</sup>
3	Hexadecane.	4.03	0.868	Antioxidant, antimicrobial. <sup>33</sup>
4	cis-Vaccenic acid.	1.523	7.24	Antimicrobial, Antioxidant
5	Bicyclo[2.2.1] heptane, 2-(1-buten-3-yl).	14.57	1.888	Antimicrobial activity.
6	n-Hexadecanoic acid (Palmitic acid).	19.54	1.172	Flavouring Agents, Anti-inflammatory agents, Antibacterial. <sup>32</sup>
7	Tetradecanoic acid, ethyl ester.	19.66	23.337	-
8	9-Hexadecenoic acid (Palmitelaidic acid).	0.198	0.347	It improves insulin sensitivity in the whole body. <sup>34</sup>

acid which is an anti-inflammatory, antifungal and antibacterial agent<sup>31</sup> are found to be higher (19.54%) in soxhlet extract than UAE (1.17%). Trans-13-Octadecenoic acid which is Cancer preventive, hypocholesterolemic, Anti-inflammatory<sup>32</sup> are found to be significantly higher in UAE (0.97%) than soxhlet (0.38%). The presence of bioactive compounds in nopal fruit peels indicates its pharmacological behaviour. The component - (+)-Ascorbic acid 2, 6-dihexadecanoate identified is an essential nutrient responsible for maintaining good health. Though there are differences in results of both extraction techniques, the possible reason could be either breaking of the weaker bonds present in complex molecules into simpler structures and/or denaturing of heat sensitive bioactive compounds at a higher temperature and a longer time. Moreover selection of solvent with different extraction method also plays a major role since highly polar solvents have a high extraction yield. The results may vary for each extraction method due to the impact of different extraction methods from various plants parts. Figure 4 shown

below indicates the GC-MS chromatogram for soxhlet extraction of nopal fruit peel.

## CONCLUSION

Conventional (soxhlet) extraction and Ultrasound-Assisted Extraction (UAE) techniques were used to extract colorant from nopal fruit (*Opuntia ficus-indica*) peel. UAE process variables were optimized for BC and BX using RSM. The effect of independent process variables of UAE were studied using BBD. Soxhlet extraction of colorants (BC and BX) from nopal fruit peel was found to yield 4.51 mg/g of BC and 0.67 mg/g of BX. Whereas the maximum extraction yield of BC and BX for UAE from *Opuntia ficus-indica* peel were determined at 16 min, 40°C, 1/22 g/mL, the yield was found to be  $3.00 \pm 0.23$  mg/g BC,  $0.225 \pm 0.02$  mg/g BX. The better yield of UAE with a shorter extraction time of 16 min compared to soxhlet (8 hr) could be attributed to the acoustic cavitation of ultrasound. GC-MS analysis of nopal fruit peel revealed that ethanol extracts of *Opuntia ficus-indica*



fruit peel revealed two fold higher results of (+)-Ascorbic acid 2, 6-dihexadecanoate of UAE than Soxhlet. Also other phytochemicals such as n-Hexadecanoic acid (Palmitic acid) which possess anti-inflammatory, antibacterial and antifungal activity are found to be higher. However usage of solvents with different extraction method also plays a major role since highly polar solvents have a high extraction yield. Results reveal that conventional method is time consuming and uses excess amount of solvent. Therefore, ultrasound-assisted extraction process may be considered as a promising technology for the enhancement of the extraction of colorants from red nopal fruit (*Opuntia ficus-indica*) peels since it reduces the extraction time, solvent consumption and enhances extraction yield.

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## CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

## ABBREVIATIONS

**UAE:** Ultrasound-assisted extraction; **SEM:** Scanning electron microscope; **GC-MS:** Gas chromatography and mass spectroscopy; **BC:** Betacyanin content; **BX:** Betaxanthin content; **mg/g:** Milligram per gram; **dW:** Dry weight.

## SUMMARY

- Nopal fruit (Prickly pear) peels were used to extract the natural colorants (Betacyanin and Betaxanthin).
- Conventional Soxhlet and Ultrasound-Assisted Extraction (UAE) techniques were employed.
- UAE given the better yields at shorter time and minimum solvent consumption compared to Soxhlet method.
- The UAE crude extract contains about 69% of (+)-Ascorbic acid 2 and minor components such as 6-dihexadecanoate, and palmitic acid which possesses anti-inflammatory, antibacterial, and anti-fungal activities.

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