Chapter-5

To extract the bioactive compounds from fresh and dried fruit using various green and novel technologies and their combination

5.1. Introduction

Natural herbal antioxidants derived from a variety of fruits are currently gaining popularity due to their potential health benefits. An extraction process can be used to get the bioactive components from *T. chebula* for usage in functional foods or nutraceuticals. For the extraction of phenolic mixtures from haritaki, many extraction processes have been used, including a reflux framework combined with water-ethanol and water-propylene glycol, and subcritical water extraction [1, 53, 56]. In any case, standard extraction processes diminish the antioxidant activity of natural compounds due to high temperatures and extensive treatment times. Ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), enzyme-assisted extraction (EAE), and other novel methods are among them. These developing technologies, as compared to traditional processes, can dramatically increase yields, improve efficiency, minimise solvent and energy usage, and are ecologically benign. These innovative technologies are now being researched at the university level and are progressively being used in the food business [17].

In comparison to traditional procedures such as hydrodistillation, steam distillation, and solvent extraction, supercritical carbon dioxide ($scCO_2$) extraction has been proven to be a superior way for extracting aromatic lipophilic chemicals from natural resources. The use of safe solvents, moderate temperatures to minimise thermolabile chemical degradation, the ability to alter selectivity, improved resource utilisation, and increased extraction efficiency are all features of this extraction technique [63].

The advantages of using supercritical fluid extraction to save process energy, minimise labour, and enhance shelf life have been highlighted by a number of publications. Supercritical CO_2 extraction is a green, new technology for selective extraction of lipophilic, non-polar, or mildly polar chemicals that has been successfully used for related molecules [30, 57].

Thermal pre-treatments, or treatments in which the temperature is raised throughout the process, can cause component deterioration, thus they must be optimised to avoid this. For plant materials with a compact cellular wall, mechanical pre-treatments are sufficient. Soaking the material in H_2O and ethanol can also weaken the cellular structure and make solvent penetration easier, but it takes time [10, 22, 64]. Microwave radiation n is a procedure in which energy is directly transferred to plant material, creating

molecular interaction with the electromagnetic field. It provides homogeneous heating in a relatively short time this manner. This improves the porosity of the material, making it easier for the solvent to penetrate and the chemicals to be available [52]. Given that microwave radiation involves the release of energy that is largely absorbed by plant moisture content, combining moistening and microwave exposure can result in a considerable increase in material permeability in a short period of time [16]. Enzymatic pre-treatment is a biodegradation process that breaks the cellular wall in moderate circumstances. This preparation can increase the amount of oil released by the plant [36]. The enzymatic pre-treatment also has the benefit of not requiring complicated equipment [34].

There are few reports on combining different treatments for extraction of bioactive compounds from plant materials like hemp [61] and flowers such as *Clitoria ternatea* and *Hibiscus rosa sinensis* [29] or during vinification [46] there have been no reports where the novel technologies like ultrasound, microwave or enzyme treatments have been used as pre-treatments before subjecting the plant material to supercritical fluid extraction.

There have been only limited research combining several procedures to extract bioactive components from pulp. In this chapter, three different pre-treatments (Ultrasound, microwave and enzymatic) have been studied individually as well as in combination to elucidate their effect on the extraction of bioactive compounds; then selecting the best combination, and see the effect of supercritical fluid extraction on yield of bioactive compounds using pre-treated pulp in comparison to untreated pulp.

5.2. Materials and Methods

5.2.1. Materials

In the month of February and March 2021, mature haritaki fruits were taken from the horticulture section of Tezpur University, Assam, India. The chemicals utilised in this study were of analytical grade and obtained from Merck-Sigma and Himedia, India.

5.2.2. Drying of haritaki pulp using different approaches

In the present study, the haritaki pulp was dried by means of various drying techniques such as tray drying (TD), vacuum drying (VD) and freeze drying (FD). First, the pulp was dried in a laboratory tray dryer (Labotech, BDI-51, B. D. Instrumentation, Ambala, India) at 40℃. The dried pulp was ground and sieved through 100 mesh sieves

followed by packing in aluminum laminated polythene bags till further use. Second, the pulp was dried using a laboratory vacuum dryer (Jeio Tech, OV-11, Korea) at 40°C. The dried pulp was ground and sieved through 100 mesh sieves followed by packing in aluminum laminated polythene bags till further use. Third, the pulp was dried using a laboratory freeze dryer (Lyolab-3S, Lyophilization Systems India Pvt Ltd.) at (-80°C). The dried pulp was ground and packed in aluminum laminated polythene bags till further use. **Fig. 5.1** depicts the fresh and dried sample obtained from different drying techniques.

(a) **(b)** **(c)** **(d)**

Fig. 5.1. Fresh and dried sample obtained from different drying techniques (a) Fresh fruit pulp (b) Freeze dried (FD) fruit pulp and powder (c) Vacuum dried (VD) fruit pulp and powder (d) Tray dried (TD) fruit pulp and powder

The dried counter parts were used for the extraction of bioactive compounds with the help of Supercritical fluid extraction (SFE) as per Jha and Sit [24] using supercritical fluid extractor vessel (Applied Separations, USA) and the extracts were collected in glass tubes in a separator. The SFE conditions used for the extraction of phytochemicals were optimized as similar to our previous study [24].

5.2.3. Pre-treatment optimization of extraction parameters using SFE

Optimization of supercritical fluid extraction parameters for tray dried haritaki powder was carried out. At the optimized condition, the fresh, freeze-dried, and vacuum-dried pulp powder were also treated [24] depicted in **Fig. 5.2**. The ratio of ethanol extract and water was 50:50. Based on the phytochemical studies, the best one among the fresh haritaki pulp, freeze-dried, vacuum dried, tray dried haritaki powder was selected for

further study. Different pre-treatment such as microwave, ultrasound, and enzymatic at two different levels of each was performed for the best one. All the extraction was carried out using distilled water in pre-treatments. Thereafter, based on their phytochemical properties, the best one from each of the treatments was chosen for further study. The best from the two levels of each pre-treatment was taken for combination as shown in **(Fig. 5.2)** and studied to observe the combined impact of these pre-treatments on the phytochemical compounds.

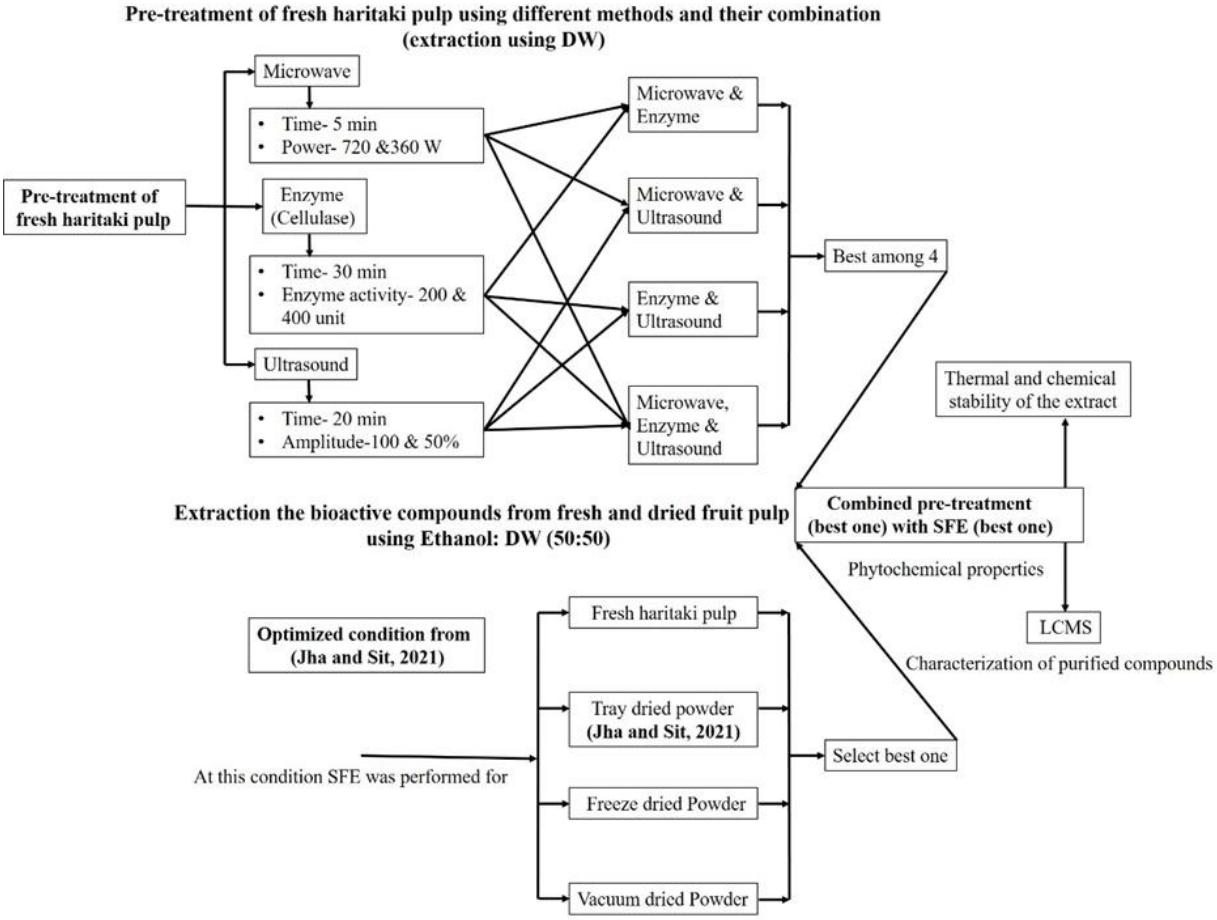


Fig. 5.2. Experimental overview of pre-treatment process of haritaki pulp

The obtained extract was analysed from the combined treatment and the best one was selected. The best pre-treatment or combination of pre-treatment was subjected to SFE (at the optimized condition). The final extracted sample was subjected to the identification of various bioactive compounds using LCMS and also used to study the thermal and chemical stability.

5.2.3.1. Novel techniques for Pre-treatment

5.2.3.1.1. Ultrasound-assisted extraction (UAE)

Haritaki sample (5 g) was extracted with distilled water (250 mL). In the UAE procedure, the sonicator (Sonics & Materials, INC. Newtown, CT, U.S.A.), model no (VCX500) with an amplitude of 50 and 100% at 20 min. The samples were centrifuged at 4,000 rpm for 30 mins at 4 °C with filter paper after being sonicated at room temperature for 30 min.

5.2.3.1.2. Microwave assisted extraction (MAE)

For the MAE, 5 g of the sample was placed in a 250 mL beaker with distilled water was placed inside a microwave (195, G.I.D.C, Industrial Estate, Makarpura, Gujarat, India), model no (HAMW/BATCH/350/1000-TZU-002). The time duration was constant (5 min) and microwave power setting were 360 and 720 W used for pre-treatment of haritaki pulp.

5.2.3.1.3. Enzyme-assisted extraction (EAE)

Cellulase from *Aspergillus spp.* was mixed with plant material and incubated at 37 ℃ for 30 min.

5.2.3.2. Combination of techniques for Pre-treatment

5.2.3.2.1. Microwave assisted -Enzymatic extraction

The combined extraction step involved a microwave-assisted extraction process together with the addition of an enzyme treatment for fresh pulp. In the case of MW+E, the combine was done with the best results obtained individual from microwave and ultrasound. The resultant pulp was characterized for bioactive compounds such as TPC, TFC, DPPH activity, FTIR and color was also done.

5.2.3.2.2. Ultrasound assisted -Microwave extraction

In the case of US+MW, the combine was done with the best results obtained individual from ultrasound and microwave. The treated pulp was characterized for physical properties, bioactive compounds and spectral characteristics.

5.2.3.2.3. Ultrasound assisted - Enzyme extraction

In the case of US+E, the combine was done with the best results obtained individual from ultrasound and enzyme. The treated pulp was characterized for physical properties, bioactive compounds and spectral characteristics.

5.2.3.2.4. Ultrasound- Microwave- Enzyme assisted extraction

In this extraction, the US+MW+E, combine was done with the best results obtained individual from ultrasound, microwave and enzyme. The treated pulp was characterized for physical properties, bioactive compounds and spectral characteristics.

5.2.4. Extraction and characterization of bioactive compounds using SFE

The best method obtained from the pre-treatment experiments was subjected to supercritical fluid extraction to further increase the efficiency and yield of extraction.

Table 5.1. Optimized condition of SFE

Flow rate (mL/min)	Pressure (bar)	Temperature (ºC)	Time (min)
3.34	166.94	51.97	67.47

The bioactive compounds were extracted using SFE as per the condition detailed in our previous study [24] (**Table 5.1**). The extract was characterized for pH and thermal stability. In addition, the extract of pre-treated using combined microwave and ultrasound fresh haritaki pulp was subjected for LC-MS.

5.2.5. Determination of bioactive compounds

The total phenolic content was calculated as discussed in section 3.2.8.2, the total flavonoid content was calculated as discussed in section 3.2.8.3, and the total antioxidant activity was calculated as discussed in section 3.2.8.4.

5.2.5. Fourier transform infrared spectroscopy (FT-IR)

The FTIR Spectrophotometer was used to record the FT-IR spectra (Thermo Nicolet Model: 6700, UK). Before the measurement, the materials were mixed with KBr and formed into pellets. The apparatus was standardised using a KBr pellet as a blank, and the spectra were obtained in the 400-4000 cm^{-1} range [2].

5.2.6. Stability of pre-treated fresh haritaki extract

5.2.6.1. Effect of pH on the stability of bioactive compounds

The effect of pH on bioactive compounds of the haritaki pulp extract was studied as reported by Sun et al. [59]. The pH value of the extract was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 with buffer solutions. Then, the effect of pH on bioactive compounds of extract were determined in a similar way as discussed above.

5.2.6.2. Effect of heat treatment on the stability of bioactive compounds

The effect of heat on the bioactive compounds of the haritaki pulp extract was studied, as reported by Krungkri and Areekul [32]. The extract was incubated at 55, 65, 75, and 85 °C in the water bath for 60 min, followed by estimation of the effect of heat on bioactive compounds in a similar way as discussed above.

5.2.7. Liquid Chromatography Mass Spectrometry (LC-MS)

The pre-treated fresh haritaki extract was characterized using LC-MS as per the method of Elavarasi et al. [15] with some modification. Separations were performed on an MSpak GF-310 4E column (250×4.6 mm, 3 m). Formic acid (0.2%) in water (A) and acetonitrile (B) are the mobile phases. The injection volume was 20 µL, the flow rate was 10.0 mL min^{-1}, and the monitoring wavelength was 217 nm. A gradient solvent system with the following solvent concentrations was used: 0-2 min, 10% B; 2-17 min, 35% B; 17-25 min, 10% B; and 25-30 min, 10% B. The injection volume was 20 µL, and the flow rate was 10.0 mL min^{-1}. An ESI probe was used in the MS analysis of the LC effluent. Negative ion mass spectra were generated by using the Bruker microTOF-Q system with the capillary temperature set to 300 °C and the ESI spray voltage set to 4 kV. The retention duration, ESI MS, and UV max of phytoconstituent peaks were compared to published literature data to identify them.

5.2.8. Color properties

The color properties were calculated as discussed in section 3.2.9. Total color difference (ΔE^*) were calculated using following equation:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (5.1)$$

5.2.9. Statistical analysis

The data obtained was analysed using Armonk, NY: IBM SPSS Statistics Version 20.0. Origin Pro 8.5.0 SR1 was used to create the graphs (Origin Lab Corporation). The obtained data was analysed using DMRT test at the significance level of 5%.

5.3. Results and Discussion

5.3.1. Impact of drying techniques on the extraction of bioactive compounds

Fresh haritaki pulp had TPC, TFC and DPPH of 497.02 mg GAE/ g, 172.65 mg QE/g and 93.39% inhibition respectively **(Fig. 5.3)**. Fresh and dried samples were used to extract the bioactive compounds using supercritical fluid extraction. During drying of pulp, the bioactive contents were influenced which are related to the duration and intensity of heating. Freeze drying of pulp result in TPC of 474.69 mg GAE/g, 168.27 mg QE/g of TFC and 93.17% of DPPH activity. Freeze drying of pulp result in the maximum retention of bioactive compounds when compared to the vacuum and tray drying. Shofian et al. [58] reported the TPC value for freeze dried tropical fruits such as acidic starfruit (142.9 mg GAE/100 g FW) and sweet starfruit (209.9 mg GAE/100 g FW), mango (56.0 mg GAE/100 g FW) and papaya (57.6 mg GAE/100 g FW). Mitra et al. [41] investigated the extraction of Coumarin where the highest yield of coumarin (90.13±0.11 μg/g) was observed at 55°C for 150 min under 24,805 kPa. In the supercritical fluid carbon dioxide extraction, the results demonstrated that the coumarin yield generally increased with time, temperature (35-55°C), and pressure (19,980-24,805 kPa). Cobb et al. [8] investigated the extraction of Lutein where temperature of 40°C, pressure of 6820 psi, and ethanol addition of 15% by volume were the ideal conditions. Lutein extraction was 2.6 times more under ideal SC-CO_2 processing conditions than it was under solvent extraction of the Corn Gluten Meal. To recover and successfully extract phenolic components from haritaki, supercritical fluid extraction is advised. Therefore, the supercritical fluid extraction may boost the potency of the polyphenols and other medicinal components in haritaki.

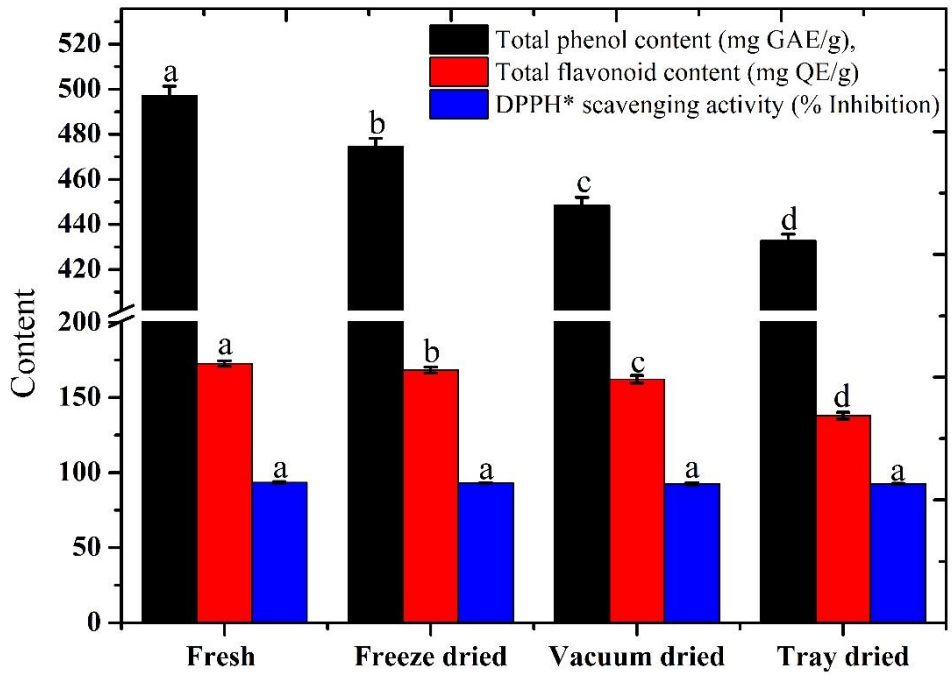


Fig. 5.3. Impact of fresh and various drying treatment on bioactive compounds of haritaki pulp

The reported values are comparatively less than the values reported in present study. Despite the efficiency of freeze-drying, bioactive chemicals were shown to be somewhat degraded in this investigation. The decrease in phenolic compounds after freeze-drying could be linked to cellular decompartmentalization during the pre-freezing step [5], followed by phenolics reacting with proteins during the dehydration process, which could affect their extractability [39]. Vacuum drying of pulp result in slight decrease of bioactive compounds. TPC and TFC was reduced by 10.65 and 5.83% in comparison to fresh counterparts. DPPH activity was also affected by vacuum drying where the 2% of reduction was observed. In case of tray drying, the levels of bioactive compounds were substantially lowered than the above-mentioned techniques. Enzymatic reactions involving polyphenol oxidases may be to blame for the loss of bioactive during tray drying [37]. Additionally, it has been demonstrated in some instances that longer drying times cause enzymatic browning, which increases losses in non-blanched tissues [27, 40].

From these experiments, it was observed that, drying methods have substantially reduced the bioactive compounds present in the pulp in comparison to the fresh counterparts. Thus, for further study, the fresh haritaki pulp was selected for pre-treatment, extraction of bioactive compounds and stability against pH and temperature.

5.3.2. Pre-treatment of haritaki pulp

By allowing and/or encouraging the release of active compounds, pre-treatment methods aid in the breakdown of cell walls and reduces the material's internal resistance, resulting in an increase in the solvent's availability of the target components. By doing so, the extraction process can be sped up and material exploitation made more effective [62]. We subjected haritaki pulp to the processes of ultrasound, microwave, enzymatic hydrolysis, and a combination of these techniques to observe the effect of individual and combined effects on the extraction ability of bioactive compounds from haritaki pulp (**Fig. 5.4**).

5.3.2.1. Effect of novel techniques on extraction of bioactive compound of haritaki

5.3.2.1.1. Ultrasound-assisted extraction

UAE was applied at a two different amplitude such as 50 and 100%. At 50% TPC, TFC and DPPH activity of treated pulp were observed to be 305.77 mg GAE/g, 98.02 mg QE/g and 89.51% respectively (**Fig. 5.4**). On the other hand, when the amplitude is set to 100%, the bioactive content is reduced. Even though the composition of the extracts was different, the effects of ultrasonic cavitation and heating on the ability of chemicals in the plant matrix to dissolve in water were the same. In terms of the overall water solubilization capacity of the chemicals contained in the plant matrix, the effects caused by ultrasonic cavitation and heating were equivalent, despite variances in extract composition. As previously stated, the pre-treatment in the US improved the mass transfer capacity of bioactive chemicals. Pre-treatment in the US increased total solid leaching, and heat treatment softens plant tissue, reducing cell wall integrity and bond hydrolysis of phenolic chemicals associated to the carbohydrate-lignin complex, increasing phenol and other component solubility [7, 11, 60]. Egüés et al. [14] similarly showed a decline in bioactive chemicals with increasing amplitude, claiming that a 50% ultrasonic amplitude allows for maximal bioactive compound extraction. This appears to be cavitation, which happens as a result of an ultrasonic wave compression and rarefaction cycle. The amplitude of the ultrasonic waves determines the compression and rarefaction. As a result, the higher the ultrasonic amplitude, the less cavities are created, resulting in a less extraction yield, due to the constant collapse of the micro-bubble, the structure of the pigment and polyphenols was damaged at high amplitude and its stability deteriorated [14].

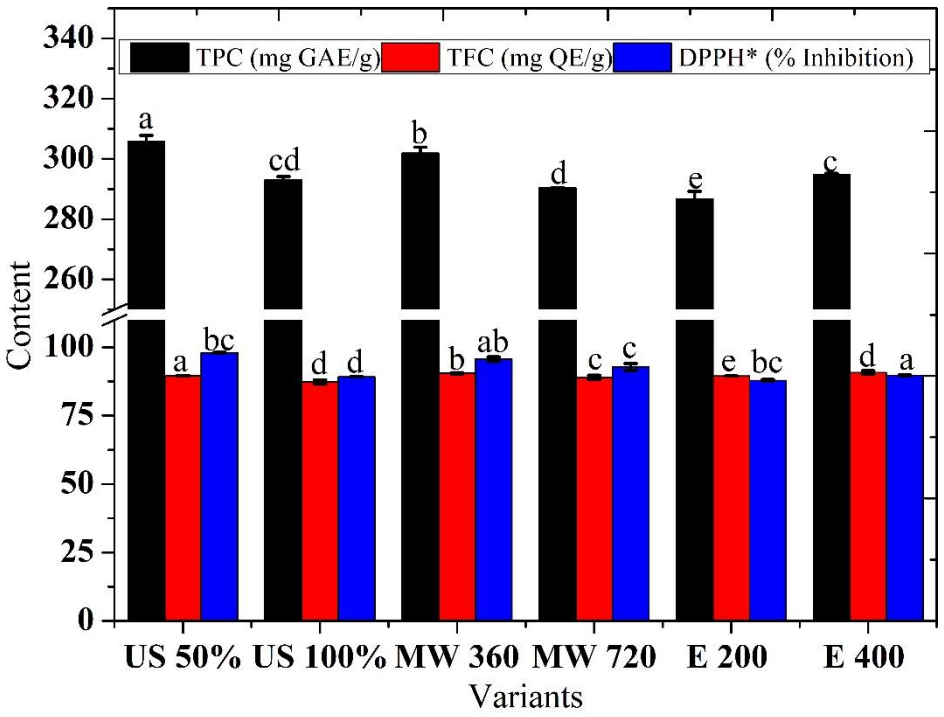


Fig. 5.4. Effect of various pre-treatment treatment on bioactive compounds of haritaki pulp

Researchers Wang and colleagues [66] looked on polyphenol extraction. The outcome demonstrates that the water CO_2 system can increase the polyphenols and active ingredients in apple peels with UAE. However, the current work aims to highlight the significance of cutting-edge green pre-treatments like ultrasound treatment.

5.3.2.1.2. Microwave-assisted extraction

Microwave pre-treatment is distinguished by the fact that its energy is immediately delivered to the plant material as a result of the interaction between the plant material and the electromagnetic field. As a result, homogeneous heating of the material is obtained during the operation, resulting in improved extraction. MAE was applied at two different power such as 360 and 720 W which result in varied effect on the bioactive compounds. At 360 W, TPC, TFC and DPPH activity of haritaki pulp were 301.91 mg GAE/g, 95.68 mg QE/g and 90.49%respectively, whereas at 720 W, 290.45 mg GAE/g, 92.76 mg QE/g and 88.96% of TPC, TFC and DPPH activity respectively, were observed (**Fig. 5.4**). These findings matched those of prior research on *Eucalyptus robusta* [3]. Gallic acid, catechin, and tannic acid were extracted from grape seeds (*Vitis vinifera*) by Krishnaswamy et al. [31], who discovered that the response variables were maximum for 6 min of MAE of grape seed (GS) with 32.6% ethanol at 121 W and a desirability function

of 0.947. In terms of mg of GAE, CAT, and TAE per gram of GS, the expected extraction yields were 13±0.89, 21.6±1.59, and 15.9±1.32 respectively. For the suppression of DPPH, the anticipated antioxidant activity per gramme of dry weight GS was 80.9%. MAE and CSE of the phenolic contents from Sea Buckthorn (*Hippophae rhamnoides*) were compared by Périno-Issartier et al. [49]. When compared to CSE extract (741 mg GAE/g), MAE extract had significantly higher phenolic contents (1,147 mg GAE/g), as well as stronger antioxidant activity as measured by the DPPH assay. Compared to conventional solvent extraction with MAE, according to Pap et al. [48]. The results revealed that by using an optimised MAE procedure, the anthocyanin yield was enhanced by 20%, and the extraction time required to achieve the maximum yield was decreased from 300 to 10 min. At 4.73 min, with a recovery of 57.0%, MAE was seen to be successful in achieving maximal polyphenols from Cranberry Pomace [12]. The extraction efficiency decreased when the power was increased, which may be explained by the degradation of proanthocyanidins at higher temperatures generated by the greater power.

5.3.2.1.3. Enzyme-assisted extraction

When plant material was exposed to cellular wall degradation enzymes, the material's structures were damaged and hydrolyzed, allowing the solvent to penetrate and the components to be available. In the present study, cellulase enzyme was used in two different unit i.e., 200 and 400 for 30 min. During pre-treatment with enzyme, haritaki pulp treated with 400 U result in higher bioactive compounds than 200 U. TPC, TFC, and DPPH activity were found to be 294.80 mg GAE/g, 89.69 mg QE/g, and 90.74% of inhibition respectively when treated with 400 U, while 286.71 mg GAE/g, 87.85 mg QE/g, and 89.49% of inhibition, respectively, in case of 200 U (**Fig. 5.4**). However, the difference between both treatments were not so significant in terms of bioactive compounds. In general, enzyme pre-treatment increased the recovery without affecting its chemical attributes. One probable explanation is that in the early stages, with more enzyme (400 U), the cell wall was destroyed more efficiently, allowing more bioactive compounds to be released. However, if the enzyme concentration is low, the enzyme response would not achieve equilibrium and halt decreasing enzymatic hydrolysis, resulting in a decrease in bioactive compounds. As a result, we determined that 400 U was the best quantity of enzyme combination for this investigation. The outcome demonstrates that extracts treated with Tea Leaf Enzyme Extract (TLEE) had higher

vanillin concentration (4.2%) than extracts treated with Viscozyme extract (2.4%) [43]. The peels were pretreated with cell-wall-degrading enzymes and solvent extracted to improve lycopene recovery. The outcome reveals that an average of 25 kg/ton of oleoresin with a 6.8 weight percent lycopene content was produced [69]. It is proposed that EAE extraction is a good and effective approach for recovering phenolic compounds from haritaki, as well as for extracting the heat-resistant bioactive chemicals.

5.3.2.2. Effect of combined techniques on extraction of bioactive compound of haritaki

From the abovementioned pre-treatment, haritaki pulp treated with ultrasound-50% amplitude, microwave-360 W and Enzyme-400 U result in the maximum recovery of bioactive compounds. Thus, these techniques at specified conditions were selected for combined treatment to study their collective impact on the extraction efficiency of bioactive compounds of fresh haritaki pulp.

5.3.2.2.1. Microwave assisted -Enzymatic extraction

Microwave application and enzyme addition were tested in a combined extraction experiment to see whether they had any synergetic effects on enhancing bioactive component production. In recent years, combining innovative approaches like microwave-enzyme assisted extraction [18] and ultrasound-enzyme assisted extraction [65] has emerged as a popular choice in extraction investigations.

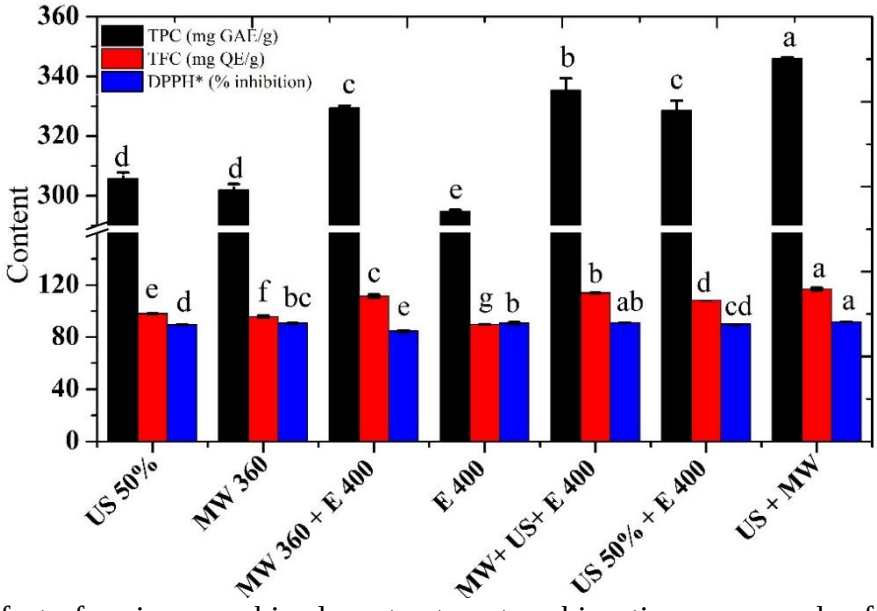


Fig. 5.5. Effect of various combined pre-treatment on bioactive compounds of haritaki pulp

Haritaki pulp treated with enzyme (400 U) for 30 min which was further treated with microwave (360 W) for 5 min. The resultant pulp was characterized for bioactive compounds and was observed that, TPC value was 329.37 mg GAE/g, 111.48 mg QE/g of TFC and 84.54% of DPPH activity was seen (**Fig. 5.5**). The increase of the bioactive compounds after applying the combined treatment can be caused by the hydrolysis or the pulp mucilage, which can release fragments with greater antioxidant capacity than the polymers from which they originate [6], or by the polymer networks break down, which releases antioxidant compounds, including phenolic compounds [33]. The values are higher than alone enzymatic treatment. In addition, the effects of microwave aided extraction result in antioxidant component content in haritaki pulp. The similar result was reported in sesame bran where the combined effect of microwave and enzyme improved the protein and antioxidant capacity [19].

5.3.2.2.2. Ultrasound assisted - Microwave extraction

In the case of US+MW, the pulp was treated with ultrasound at 50% of amplitude for 20 min followed by microwave treatment for 5 min at 360 W. The treated pulp had TPC value of 346.05 mg GAE/g, 116.93 mg QE/g of TFC and 91.57% of DPPH activity. The obtained result in this combination is higher among all the combined treatments (**Fig. 5.5**). The absorption of ultrasonic and microwave energy increased, causing the temperature of the extraction system to keep rising and speeding the diffusion rate of haritaki's bioactive components, which may be linked to the strength of ultrasonic and microwave waves. Pang et al. [47] reported that the impacts of various operational factors, such as ultrasonic power, microwave power (240 W), and microwave duration (10 min), result in a shorter extraction time and greater efficiency, which might be attributed to the effect of combined impact on disrupting the paprika cellular structure.

The target molecules in the sample align in a directional manner as a result of the microwave electromagnetic field's action, and high frequency oscillation occurs with the alternate change of the microwave electromagnetic field, accelerating the bioactive of haritaki compound from solid phase to solvent phase and cutting processing time, according to the results of this activity [4]. The extraction of proteins from coffee green beans was explored using a combination of innovative extraction techniques. It has been demonstrated that the ultrasound-assisted extraction (UAE) method has no effect on the protein quality, whereas the microwave-assisted extraction (MAE) method exhibits some

degradation as a result of the high temperatures obtained. The findings show that to maintain the protein fraction's quality during alkaline extraction, rigorous temperature control is necessary [50]. The findings of this study demonstrate for the first time that UAE and MAE treatments boosted yield in addition to phenolic composition of haritaki. Additionally, it offers a solution and a different approach to enhance the commercial usage of haritaki in the creation of functional foods. This is due to the low cost and scalability of the methodologies utilised in this study, which can be applied to the commercial sector.

5.3.2.2.3. Ultrasound assisted - Enzyme extraction

The treated pulp had TPC value of 328.64 mg GAE/g, 108.14 mg QE/g of TFC and 89.85% of DPPH activity (**Fig. 5.5**). For enhancing phenolic chemicals, a combination extraction technique was considered. US boosted both the extraction of anthocyanins and tannins by 7 and 16%, respectively, while enzyme treatment alone increased tannin concentration by 13%. The outcomes of the treatments individually were not enhanced by the addition of US and enzymes, both of which were applied at the start of the maceration period [46]. The most intriguing result was seen when both treatments were combined to increase the commercial use of haritaki in functional food composition. This is due to the low cost and scalability of the methodologies utilised in this study, which can be applied to the commercial sector. Puri et al. [51] claim that when enzymatic treatments are used, partial hydrolysis of the mucilage allows for the extraction of chemicals attached to the polymeric matrix, making sample processing easier. Galacturonic acid is a common mucilage molecule that may be produced during enzymatic hydrolysis; nevertheless, exposure to functional groups (hydroxyl and carboxyl) can raise total phenols levels [9]. Additionally, there were no appreciable increases in the overall concentration of phenolic compounds as a result of ultrasonic wave exposure. By combining enzyme extraction with ultrasonic assistance, it is possible to get around some of the drawbacks of enzyme technology, such as increased solvent consumption and longer extraction times. Ultrasound therapy may potentially increase enzyme activity in the right circumstances [42]. Liu et al. [38] investigated that the optimal extraction of bioactive compounds from *Acanthopanax senticosus* using combined ultrasound and enzyme. The results for different polar solvent enrichments showed that the highest flavonoid, polyphenol, and saponin contents were observed in the

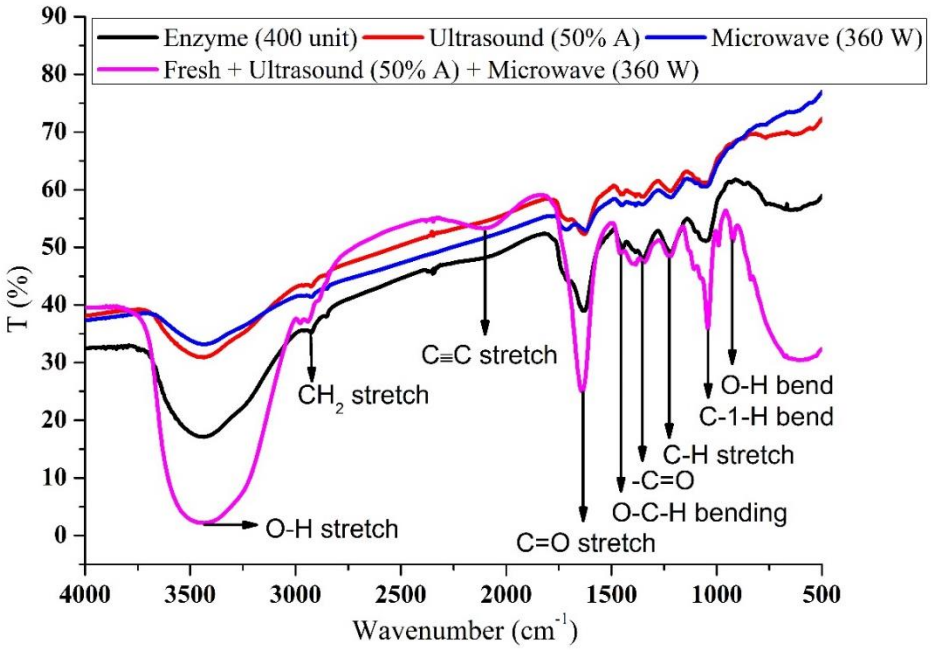
1-butanol fraction. They also documented that combined ultrasound and enzyme treatment may serve as an effective way to extract the bioactive compounds.

5.3.2.2.4. Ultrasound-Microwave-Enzyme- assisted extraction

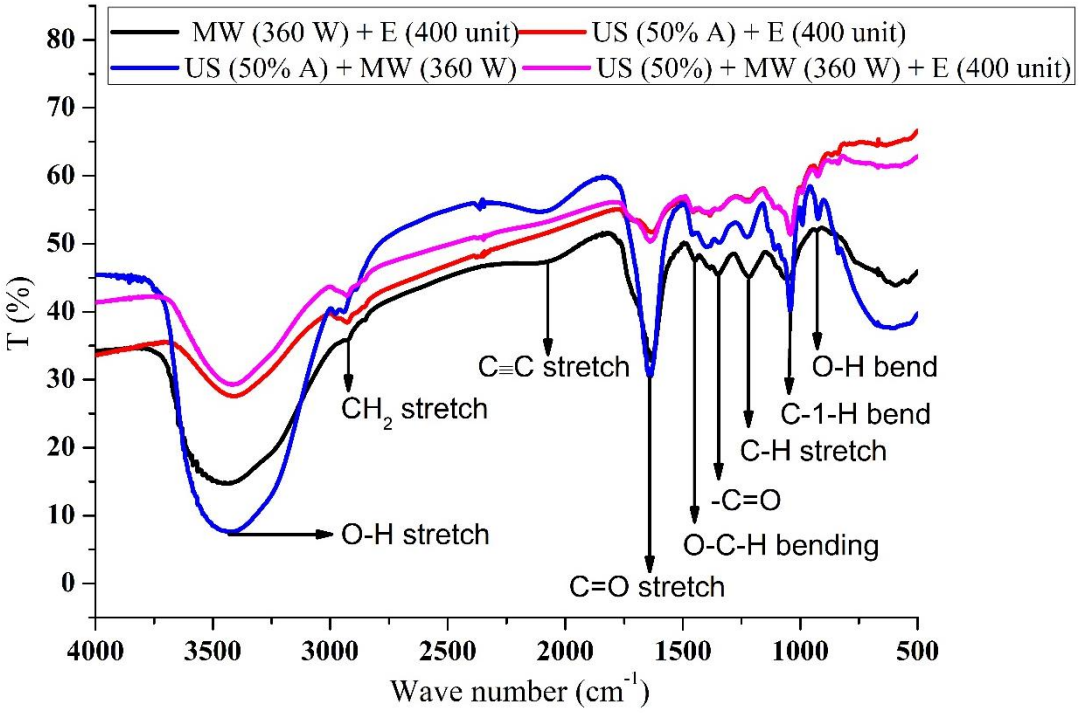
The treated pulp had TPC value of 335.37 mg GAE/g, 113.89 mg QE/g of TFC and 91.03% of DPPH activity (**Fig. 5.5**). In compared to traditional solvent extraction, the extraction of phenolic compounds from pitayas employing combination as pre-treatment is more efficient than that reported by Nayak et al. [44], who observed a 20.5% rise in citrus peels. This suggests that the impact of pre-treatments on total phenolic compound performance in extracts is dependent on the kind of vegetal material used. It was applied by Wu et al. [67] to extract antioxidant components from *Nitraria tangutorum* juice by products (NJB). It demonstrated the combination method's potential to be a powerful and effective way to extract phytochemicals.

5.3.3. Spectral analysis of pre-treated haritaki pulp

The treated haritaki pulp by means of various pre-treatment was subjected for FTIR analysis and is presented in **Fig. 5.6**. The characteristic broad peaks at 3351 to 3404 cm^{-1} assigned to O-H stretching of hydroxyl group was of low intensity in case of microwave treated pulp (**Fig. 5.6a**). The ultrasound treatment causes this band's intensity to slightly increase, indicating that the starch's microstructure has greater capacity to retain bound water [26].



(a)



(b)

Fig. 5.6. FTIR spectra of haritaki pulp extract **(a)** Individual pre-treatment **(b)** Combined pre-treatment

However, enzymatic treated pulp has broader peak than these two treatments. The C-H stretching of the glucose unit, which causes the later peaks at 2100 to 2065 cm^{-1}, is likewise increased by ultrasonic treatment. Peaks are attributed to the vibration of C-O stretching, C=O, C-O-H, and C-1-H, respectively, at 1647 to 1652 cm^{-1}, 1385 cm^{-1}, 1218 cm^{-1}, and 1055 cm^{-1}. Furthermore, the bands at 923 to 936 cm^{-1} that were associated with anhydrous glucose ring stretching vibrations were observed. In general, these above-mentioned stretching were intense in enzymatic followed by microwave than ultrasound treatment. From illustration **5.6b**, it can be easily observed that, the peak was sharper and more stretched in case of combined treatment of ultrasound and microwave than individual enzyme, ultrasound and microwave pre-treatment.

5.3.4. Extraction and characterization of bioactive compounds using SFE

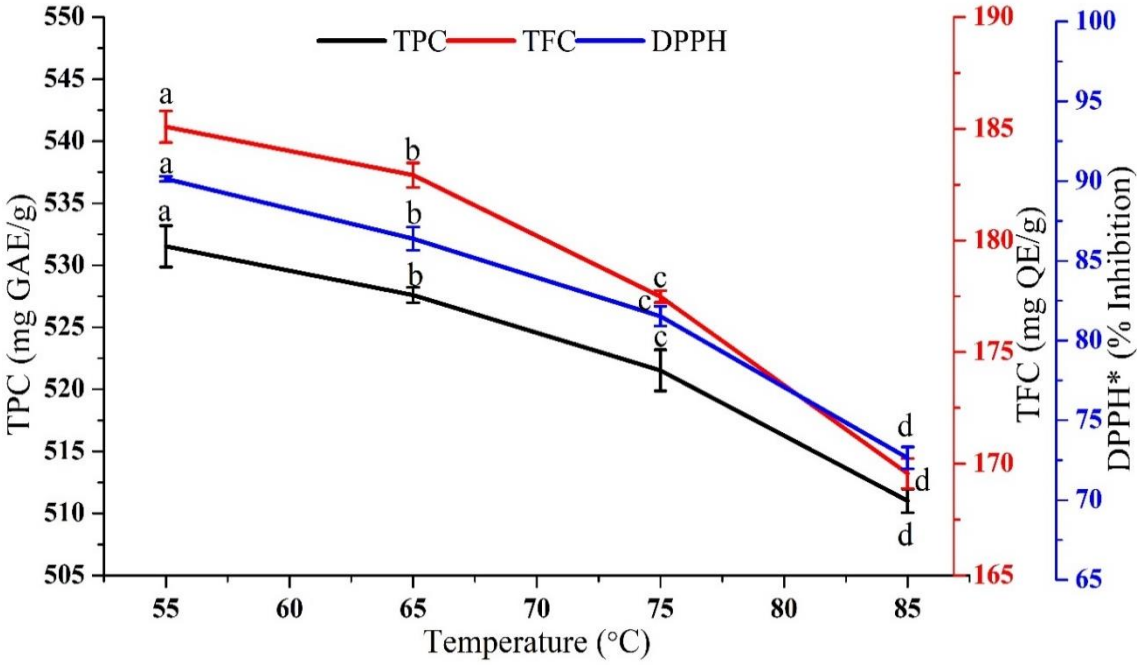
The best method obtained from the pre-treatment experiment treatment (individual and combined) was subjected to SFE. The condition of SFE used in the present study was as per the optimized condition which have been detailed in our previous study [24]. The extract after SFE and the extract from the best pre-treatments are characterized

for the pH and thermal stability. LC-MS study was also carried out to identify the phytochemical compound present in the extract from the best pre-treatment. FTIR and color properties were also assessed.

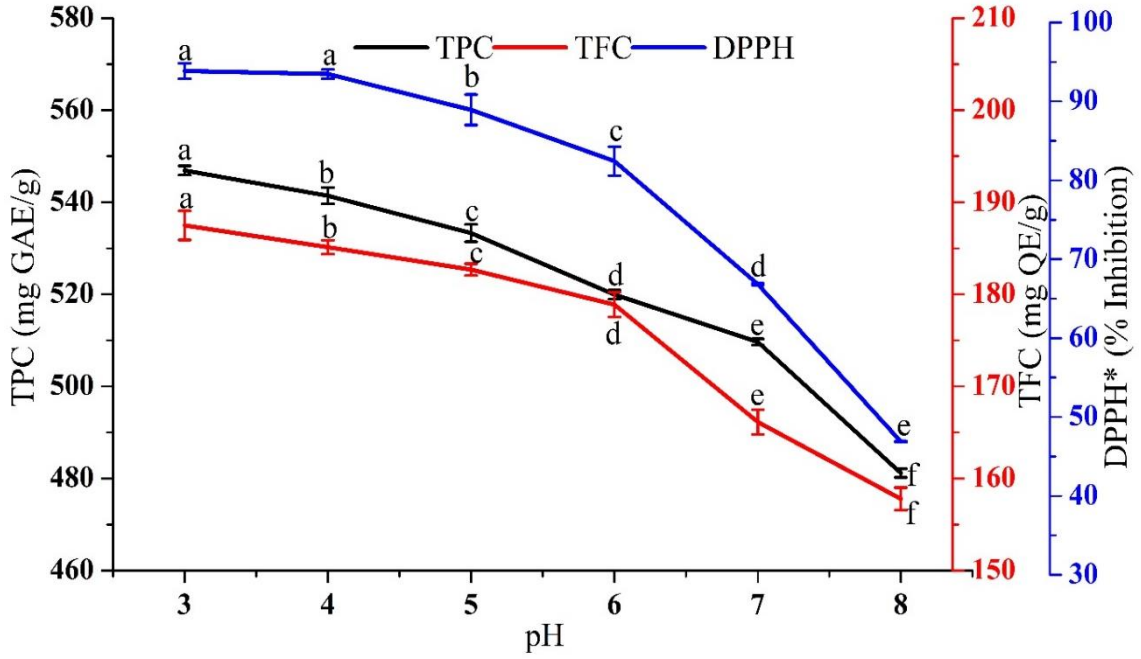
Microwave-assisted Ultrasound extraction at optimum conditions was found as an excellent technique to improve the extraction of bioactive compounds from haritaki. The pulp treated with combined techniques was allowed to extract bioactive compounds using the SFE and further characterized for TPC, TFC, and DPPH activity. It was observed that TPC, TFC, and DPPH activity of the extract were 533.91 mg GAE/g, 186.21 mg QE/g, and 93.62% inhibition respectively. The bioactive compounds were significantly higher than the other combined pre-treatment indicating the synergistic effect of ultrasound and microwave on haritaki pulp.

5.3.4.1. Effect of pH and temperature on the stability of bioactive compounds

The stability of physiologically active chemicals in plant raw materials is critical, especially when processing fruits and vegetables under harsh circumstances. In this study, the extract of pre-treated haritaki was analysed for pH and thermal stability. From the illustration **Fig. 5.7a**, it can be observed that, as the pH increases, the content of bioactive compounds were decrease. The maximum bioactive compounds were seen at pH 3 where TPC, TFC and DPPH activity were 546.90 mg GAE/g, 187.48 QE/ g and 93.84% of inhibition respectively. The acidic pH is more commonly utilised in plant material processing technologies than the alkaline pH [20]. Antioxidative bioactive substances may react with one another, resulting in unanticipated changes in antioxidant activity. The proportion of physiologically active molecules in potatoes has an impact on their antioxidant activity, according to research by Rytel et al. [54]. According to other authors [21, 35, 45], the type and quantity of certain anthocyanins as well as the presence of phenolic acids, particularly chlorogenic acid and its isomers, are all factors that affect the antioxidant activity of potatoes. The amount of free hydroxyl groups in the structure of anthocyanins, on the other hand, controls their antioxidant activity. Phenolic chemicals are an essential element of human diet, with well-known health advantages. These elements are utilised as quality indicators in a variety of food processing operations. Temperature affects the stability of these chemicals, and they are rapidly destroyed during drying.



(a)



(b)

Fig. 5.7. (a) Effect of temperature on final pre-treated best one variant of haritaki pulp extracted using SFE **(b)** Effect of pH on final pre-treated best one variant of haritaki pulp extracted using SFE

As a result, it's crucial to look at how these chemicals degrade throughout the drying process [23]. **Fig. 5.7b** demonstrates the impact of temperature on the stability of bioactive compounds where the maximum content at 55 ℃ and decreased with an increase of temperature. At 55 ℃, content of phenol was 531.53 mg GAE/g, 185.09 mg QE/g of TFC and 90.14% of inhibition was recorded. The degradation of TPC, TFC and DPPH activity was also found to be more temperature dependant. The rate of degradation also increased with temperature for all the components.When the temperature was raised from 65 to 75 ℃, deterioration rose dramatically, but when the temperature was raised even higher to 85 ℃, the degradation rate did not alter significantly. The similar result was observed in our previous study [23].

5.3.4.2. LC-MS of microwave assisted ultrasound pre-treated haritaki pulp SFE extract

LC-MS analysis showed the presence of the total 36 compounds in the pulp extract which include chebulic acid, quercetin 3-O-glucuronide, methyl 2-furoate, 1,3,6-Tri-O-galloylglucose, 2-Hydroxychromene-2-carboxylate, 2,6-Digalloylglucose, 8-Hydroxyluteolin 8-glucoside 3'-sulfate, di-trans,poly-cis-decaprenyl diphosphate, methyl N-methylanthranilate, 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5'-monophosphate, (3 beta, 19 alpha)-3,19,23,24-Tetrahydroxy-12-oleanen-28-oic acid, dihydrodeoxystreptomyci, ellagic acid etc. These compounds are reported to have biochemical properties and are important for the formulation of polyherbal products and allied products (**Table 5.2**).

Table 5.2. Components identified in extracted haritaki pulp by LC-MS analysis

Sl. No.	Compound Names	Formula	Retention time (min)
1	(+)-Chebulic acid	$C_{14}H_{12}O_{11}$	1.248
2	Gallic acid	$C_7H_6O_5$	1.604
3	Ellagic acid	$C_{14}H_6O_8$	5.561
4	Quercetin 3-O-glucuronide	$C_{21}H_{18}O_{13}$	4.711
5	Asiatic acid	$C_{30}H_{48}O_5$	11.396
6	Methyl 2-furoate	$C_6H_6O_3$	1.464

7	Fertaric acid	$C_{14}H_{14}O_9$	1.92
8	Amlaic acid	$C_{27}H_{24}O_{19}$	4.481
9	1,3,6-Tri-O-galloylglucose	$C_{27}H_{24}O_{18}$	4.789
10	Isoterchebin	$C_{41}H_{30}O_{27}$	6.056
11	Methyl 2-furoate	$C_6H_6O_3$	1.464
12	2-Hydroxychromene-2-carboxylate	$C_{10}H_8O_4$	1.161
13	2,6-Digalloylglucose	$C_{20}H_{20}O_{14}$	3.36
14	alpha-Santalal	$C_{15}H_{22}O$	1.151
15	Dambonitol	$C_8H_{16}O_6$	2.343
16	Terbinafine	$C_{21}H_{25}N$	3.732
17	Tiropramide	$C_{28}H_{41}N_3O_3$	5.375
18	Solanocapsine	$C_{27}H_{46}N_2O_2$	5.758
19	Erythromycin B	$C_{37}H_{67}NO_{12}$	6.179
20	Telaprevir	$C_{36}H_{53}N_7O_6$	6.597
21	di-trans,poly-cis-decaprenyl diphosphate	$C_{50}H_{84}O_7P_2$	6.668
22	Nafoxidine	$C_{29}H_{31}NO_2$	6.966
23	8-Hydroxyluteolin 8-glucoside 3'-sulfate	$C_{21}H_{20}O_{15}S$	7.852
24	Novaluron	$C_{17}H_9ClF_8N_2O_4$	7.94
25	Dihydrodeoxystreptomycin	$C_{21}H_{41}N_7O_{11}$	9.036
26	Sphinganine	$C_{18}H_{39}NO_2$	10.957
27	Stigmatellin Y	$C_{29}H_{40}O_6$	16.362
28	Oxymetazoline	$C_{16}H_{24}N_2O$	3.66

29	Methyl N-methylanthranilate	$C_9 H_{11} N O_2$	1.858
30	L-Arginine	$C_6 H_{14} N_4 O_2$	0.987
31	Retronecine	$C_8 H_{13} N O_2$	1.158
32	S-(Hydroxymethyl)mycothiol	$C_{18} H_{32} N_2 O_{13} S$	1.06
33	5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5'-monophosphate	$C_9 H_{15} N_4 O_8 P$	3.524
34	Oxdemetonmethyl	$C_6 H_{15} O_4 P S_2$	4.192
35	Punicacortein B	$C_{27} H_{22} O_{18}$	4.742
36	(3beta,19alpha)-3,19,23,24-Tetrahydroxy-12-oleanen-28-oic acid	$C_{30} H_{48} O_6$	9.461

5.3.5. Color properties

Color is a crucial factor in determining whether or not a product will be accepted by customers. Another quality criteria that should be retained during fruit drying is the moisture content. The impact of initial pre-treatment on colour parameters in haritaki pulp extract exposed to various pretreatments and drying processes was investigated, and the findings are presented in **Table 5.3**. The bioactive compounds of dried haritaki pulp were extracted and evaluated for color parameters where L^*, a^*, b^*, and ΔE were considered and are presented in **Table 5.3**. The fresh haritaki pulp (control) was used for extraction of bioactive compounds using SFE where the L^*, a^*, b^*, and ΔE was 25.60±0.25, -1.02±0.17, 2.44±0.25, and 0 respectively. Upon drying treatment, a significant difference in the above-mentioned values was observed which can be easily observed from the table. Tray-dried pulp had the highest ΔE value, when compared with fresh, freeze and vacuum, dried pulp extract. Due to the high temperature, the value of parameter b*, which indicates yellowness or blueness, generally increased in vacuum and tray dried pulp extracts compared to control and freeze-dried samples. It was determined in control and freeze-dried samples at 2.44±0.25 to 3.49±0.37 and in vacuum and tray dried samples at

8.96±0.17 to 9.85±0.05. The colour parameter b* value increased in all dried cranberries, according to Zielinska et al. [68]. In frozen samples, it was found to be 2.4±0.2, while in dried samples, it was found to be 4.1±0.2 to 5.4±0.2. As mentioned earlier, fresh pulp exhibited the maximum bioactive compounds and was thus selected for further pre-treatment where different green techniques were applied. In the pre-treatment, the haritaki pulp treated by enzyme at 400 unit resulted in higher total color difference values than ultrasound and microwave treatment. Microwave extracted sample showed the maximum *L** and *b** value, due to an increase in temperature.

Table 5.3. Color properties of fresh, dried and pre-treated samples

Values are means ± standard deviation of three determinations (n = 5). Values followed by different superscript letter in a row are significantly different ($p \leq 0.05$).

Sl. No.	Sample names	Sample pictures	*L**	*a**	*b**	Δ*E*
1	Fresh		25.60±0.25^{a}	-1.02±0.17^{c}	2.44±0.25^{b}	0
2	Freeze dried		26.04±1.00^{a}	-1.51±0.10^{d}	3.49±0.37^{b}	1.23±0.97^{c}
3	Vacuum dried		23.03±0.06^{a}	1.30±0.12^{c}	8.96±0.17^{b}	7.38±0.05^{d}
4	Tray dried		23.51±0.06^{a}	0.37±0.08^{c}	9.85±0.05^{b}	7.82±0.04^{d}
5	Fresh+US+MW		26.17±0.11^{a}	-1.39±0.17^{d}	4.38±0.27^{b}	2.05±0.16^{c}
6	US 50%		17.90±1.29^{a}	2.69±0.37^{b}	1.26±0.34^{b}	8.62±0.66^{b}
7	MW 360 W		27.19±1.20^{a}	3.11±2.10^{c}	9.93±0.58^{b}	8.69±1.69^{c}
8	US+MW		17.46±0.80^{a}	2.46±0.25^{b}	1.92±0.34^{b} ^{c}	8.86±0.61^{c}

9	E 400 U		14.79±1.68[a]	2.00±0.15[b]	-0.48±0.46[c]	11.39±1.40[b]
10	MW+US+E 400 U		19.45±0.60[a]	1.28±0.21[c]	3.98±0.52[b]	6.74±0.44[c]
11	US 50%+ E 400 U		17.46±1.26[a]	3.37±0.30[b]	2.11±0.71[b]	9.25±1.44[b]
12	MW 360+ E 400 U		21.22±0.04[a]	0.91±0.20[c]	4.02±0.51[b]	5.04±0.29[c]

According to Zielinska et al. [68], samples treated to microwave-vacuum pretreatment at high microwave power had the highest *L** value. In fruit, the value of parameter a*, which reflects the sample's redness in its positive form, is also an essential quality indicator. In the case of combined techniques such as US+E, the Δ*E* value (9.25±1.44) and *a** value (3.37±0.30) observed maximum than other combined treatments. While *L** value was recorded maximum in MW+E where 21.22±0.04 of the values were achieved respectively.

5.4. Conclusion

In this objective, the bioactive components of haritaki pulp were studied using a variety of drying processes and pre-treatments. Because most of the beneficial components in haritaki pulp were drastically decreased after drying, fresh haritaki pulp was used for additional pre-treatment. Ultrasound at 50% amplitude, microwave at 360 W, and enzyme at 400 U have been chosen for combination pre-treatment because they provide the most bioactive characteristics. Microwave-assisted ultrasonic extraction extracts the most bioactive chemicals of all the pre-treatment methods. The extraction of bioactive substances from the haritaki is maximised when ultrasound and microwave treatments are used together. The pulp obtained by combining microwave and ultrasound was subjected to SFE extraction and evaluated for pH and thermal stability, with bioactive characteristics decreasing as pH and temperature increased.

5.5. References

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