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EFFECT OF BATH THERMOSONICATION ON PEROXIDASE ACTIVITY AND BIOLOGICALLY ACTIVE COMPOUNDS IN *KARVAND* (*CARISSA CARANDAS*) JUICE

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ABSTRACT
Karvand (*Carissa carandas*) fruit juice was sonicated on a bath sonicator at 30˚C, 50˚C and 70˚C (at a frequency of 53 KHz) to evaluate its impact on quality hampering enzymes such as peroxidase as well as on Physico-chemical and bioactive compounds such as pH, Total soluble solids, cloud values, total acidity, total phenolics, and flavonoids. Bath thermosonication studies resulted in significant inactivation of peroxidase in 130 minutes, 100 minutes, and 80 minutes at 30 °C, 50˚C and 70˚C with negligible impact on change in pH, titratable acidity as well as on °Brix. Sonicated juice at 70˚C has been shown to significantly increase the contents of total phenolic compounds and flavonoids. This work indicates the process of sonication can be engaged as an effective tool for the treatment of fruit juice processing techniques in relation to the effective retention and enhancement of antioxidant and bioactive components.

Keywords: *Fruit juice, Enzymes, Antioxidants, Phytochemicals Phenolics, Flavonoids*

INTRODUCTION
People are increasingly aware of the importance of eating a healthy diet, and they are looking for ways to get the most nutrients from their food. Fruits and vegetables are a great source of vitamins, minerals, and antioxidants, and they are also low in calories and fat. However, the way that fruits and vegetables are processed can affect their nutritional content. Minimally processed fruits and vegetables are those that have been subjected to little or no processing. This means that they have not been cooked, canned, frozen, or otherwise preserved in a way that would remove or destroy nutrients. Minimally processed fruits and vegetables are the best way to get the most health benefits from these foods. Some fruits are amongst the most popular categories due to their wide range of availability throughout the season. Some remained unexplored due to their seasonal nature though having an enormous amount of nutrients. One such fruit is *Karvand* (*Carissa carandas*).

Karvand (*Carissa carandas*) is a species of flowering shrub in the family Apocynaceae, is very common around all Asian countries and has tremendous effective medicinal and health benefits [Singh *et al.*, 2021]. Seasonal nature restricts availability of the Karvand fruit rest of the year and hence processing plays a very important role rendering less harm to the final product quality. Various kinds of enzymes in fruit juices such as peroxidase, pectin methylesterase, polyphenol oxidase, and ascorbate oxidase led to quality loss upon storage. Inactivation of these enzymes ensures the safety of the fruit juices from quality losses such as delayed browning, retention of cloud value, and retention of vitamin C. An Important approach towards enzyme inactivation is the inactivation of most heat-stable enzymes which then ensures the effective inactivation of all enzymes present in the fruit juices. Peroxidase is one such enzyme that is considered an indicator enzyme, which means if peroxidase is inactivated all the quality deteriorating enzymes are also inactivated. For many years traditional approach of heating has been common in processing fruits and vegetable juices [Kaur *et al.*, 1999]. Traditional methods are economically efficient; however, they have limitations in retaining the nutritional contents of fruit juices. Hence, the food industries are in constant search for novel alternatives that would confer less harm to the final product while retaining the maximum nutritional attributes. Ultrasonication is one such promising novel food processing technology. Ultrasonic waves are introduced into the liquid which then creates cavitation due to pressure changes. This results in the micro-bubble formation which falls in the following compression cycles and subsequently leads to high localized temperature and pressure of up to 5000K and 50,000 kPa. As a result, the intensive local energy and elevated pressure conduct a localized sterilization effect [Dalbir *et al.*, 2017, Dalbir *et al.*, 2016].

Ultrasonication applied with temperature to enable the processing at a certain defined temperature is called thermosonication, which is observed to be effective for the minimum process since the transfer of acoustic energy to the fruit juice appears to be rapid and uniform. Sonication at a controlled temperature as that in a bath thermosonicator operates at a low controlled temperature hence prone to less harm to the product as compared to heat treatments and at the same time saves processing time (Feng *et al.,* 2011, Mason *et al.,* 1996; Piyasena *et al.*, 2003).

Thermosonication has been confirmed to be successful in the processing of blackberry juice, strawberry juice, custard apple, and orange juice [Xu *et al.*, 2023, Tiwari *et al.*, 2009; Tiwari *et al.*, 2008; Dalbir *et al.*, 2017]. Development in the quality of tomato juice [Wu *et al.*, 2008] with the use of thermosonication has been described in the literature. The peroxidase enzyme has been successfully inactivated in the thermosonication process; treated samples showed better holding of color and vitamin C in the case of watercress [Cruz *et al.*, 2008] and guava juice [Cheng *et al.*, 2007].

To our knowledge, there are no available reports on the processing techniques utilized for *karvand* juice. The effect of sonication on improving *karvand* juice quality and on the bioactive content has not been reported. Hence the present study focuses on the effect of sonication in combination with mild heat on enzymes from *karvand* fruit juice such as peroxidase and pectin methylesterase. In addition, the effect of sonication on physicochemical parameters such as pH, Brix, titratable acidity, cloud value, phenolic, and flavonoids will be studied.

MATERIALS AND METHODS

2.1 Juice preparation

Karvand (*Carissa carandas*) fruits were collected from the Western Ghats region of Maharashtra, India. Fully ripe *karvand* fruits are green in color with red ting and turn purplish to black when mature. The pulp was squeezed out from the fruit and collected. The juice was diluted by adding deionized water in the proportion of 1:5. The homogeneous mixture was obtained by blending the juice for 30 seconds in a domestic blender and stored at 4°C till further processing.

2.2 Bath thermosonication treatment

Fruit juice was processed by a Kudos HP Heating – series: 53 KHz 3 - 22.5 L Heated Sonicator. The sonicator has a constant frequency of 53 kHz and variable power output. Juice processing studies in this work was at three temperature that justifies the treatment at the cold, mildly hot, and hot temperature ranges, hence the *karvand* juice was processed at three various temperatures 30 ˚C, 50 ˚, C and 70 ˚C. Each sample was equilibrated at the required temperature and controlled by circulating water around the sample beaker in the water bath. Temperature change of sample beaker and water bath was monitored by using thermometers. *Karvand* juice (30 ml) was added to a 100 ml beaker and kept in a bath sonicator. The ultrasound was applied at a selected time and samples were collected after every minute. All the experiments were carried out in triplicate runs.

2.3 Peroxidase activity

On the Jasco V-630 spectrophotometer, a peroxidase assay was performed at 470 nm as described by Ciou *et al.*, [2011]. A substrate of 4 % Guaiacol is used and the reaction mixture includes 2.73 ml of phosphate buffer (0.05 M) of pH-7.0, 1 % hydrogen peroxide (0.1 ml),4 % of Guaiacol (0.15 ml) at 30 ˚C; the addition of 50µl of *karvand* crude extract (enzyme source) the color of the reaction mixture is changed to reddish brown due to formation of 4-hydroxy Guaiacol peroxidase activity and monitored for 2 min for every 20 s. Peroxidase activity of one unit can be defined as the change in absorbance of 0.001 min^{-1}.

2.4 Peroxidase inactivation

Inactivation studies on peroxidase at the desired bath thermosonication treatments were performed.

Peroxidase activity was checked every five minutes for inactivation and given by the formula:
Residual enzyme activity = $C_t/C_0 \times 100$
Where, C_t = peroxidase activity at time t
C_0 = peroxidase activity at time zero

2.5. pH

Using a pH meter, the pH was determined (Fischer scientific), and a refractometer (ERMA, Japan), respectively.

2.6. Total acidity (TA)

In the presence of phenolphthalein indicator (1%) the sonicated sample (5ml, without dilution) was titrated with 0.1N sodium hydroxide (NaOH). Titratable acidity was determined by the following equation and represented in the form of citric acid equivalents.

$$\% \text{ Titratable Acidity} = \frac{ml\ of\ base\ titrant \times normality\ of\ base\ acid\ factor\ (citric\ acid\ ) \times 100}{sample\ of\ volume\ in\ ml}$$

2.7 Antioxidant assay

The protocol from the guidelines of Prieto, Pineda, and Aguilar, 1999 with some modifications to estimate the antioxidant capacity of the Karvand juice blend was adopted [15]. The standard used was ascorbic acid, and the details were regarded as ascorbic acid equivalent microgram (AAE)/100 mL juice, respectively.

2.8 Cloud value

Centrifugation was done using 5 ml of *Karvand* juice at 3000 rpm for 5 minutes at 25 °C using R-4C REMI Centrifuge. The Jasco V-630 spectrophotometer is used to measure the absorbance of the collected supernatant at 660 nm.

2.9 Total flavonoid content (TFC) and Total phenolic content TPC)

Folin–Ciocalteu spectrophotometric method is used to measure the total phenolic content at 750nm [Singleton *et al.*, 1999]. 0.5 ml of Juice sample extracted in 80 % of methanol (1.5 ml) was added into 1 ml of 10 % (v/v) Folin–Ciocalteu reagent (diluted with D/W). After approximately 6 minutes, 20 % of 2.0 ml of sodium carbonate solution was added. In the water bath, the reaction mixture was kept for 60 minutes at 30 ºC, and the absorbance was measured at 760 nm. The standard used was Gallic acid, and the phenolic content was shown as µg (micrograms) of GAE (Gallic acid equivalent) per gram of sample.

Total flavonoid content of the Juice sample of 1ml was added into 0.1 ml of 10% $AlCl_3$, 1 M Sodium hydroxide and 0.3 ml of 5% Sodium nitrate. The reaction mixture was incubated for 30 minutes at 30 ºC, and the absorbance was determined at 510 nm. The standard used was Quercetin, and the flavonoid content was shown as µg (micrograms) of Quercetin equivalent per gram of sample [Lin and Tang, 2007].

RESULTS AND DISCUSSION

3.1.1 Inactivation of peroxidase activity in karvand fruit juice by bath thermosonication:

In the present work inactivation of karvand peroxidase was performed at three different temperatures 30 ± 2°C, 50 ± 2°C and 70 ± 2°C. Sonication is a process that uses sound waves to create high-pressure, low-duration pulses. These pulses can cause physical damage to enzyme molecules, which can lead to their inactivation. The combination of bath thermosonication (heating and sonication) is a very effective way to inactivate enzymes. This could be because it combines the effects of both temperature and sonication to damage the enzyme molecules. This study found that complete inactivation of karvand POD (enzyme Peroxidase) required 130 minutes at 30°C, 100 minutes at 50°C, and 80 minutes at 70°C (Table 1) This shows that the higher the temperature, the shorter the time required for complete inactivation. Guiseppi-Elie *et al.*, (2009) observed lower temperatures can have a "masking effect" on enzyme inactivation. This means that lower temperatures can make it more difficult to inactivate enzymes. This is because the enzyme molecules are less likely to vibrate at lower temperatures, which makes them less susceptible to damage.

Table 1: Inactivation studies of peroxidase enzyme

Sr. No.	Sample treatments	Time taken for 50% inactivation (Mins)	Time taken for 100% inactivation (Mins)
1	30°C	60	130
2	50°C	60	100
3	70°C	40	80

3.1.2 Effect of bath thermosonication on pH, Titratable acidity, and Total soluble solids (TSS):

Bath thermosonication has no significant effect on the pH and total acidity of karvand juice at all three temperatures studied and the data is shown in Table 2. The pH of Karvand juice of 30°C,50°C, and 70°C, and control was found to be 3.0 indicating Karvand juice is acidic in nature. The titratable acidity of was found to be 0.064%, 0.064%, 0.044%, and 0.044% for control, 30°C, 50°C, and 70°C samples respectively. Observations of Bhat *et al.,* (2011) on kasturi lime juice and Abid *et al.,* (2013) on apple juice are very much in agreement with the findings mentioned in this study. Total soluble solids (TSS) are a measure of the number of dissolved solids in a liquid, such as juice. The dissolved solids in juice are primarily sugars, with smaller amounts of other compounds such as organic acids, proteins, and minerals. Bath thermosonication of karvand juice showed no significant effect on total soluble solids (ºBrix/ Brix value) in the karvand juice (Table 2). Bhat *et al.*, 2011 observed a significant decrease in the Brix value of kasturi lime juice. However, an insignificant change in Brix value in *karvand* juice is in agreement with the observation of ultrasound-treated apple carrot juice blends [Tiwari *et al.,* 2009]. The onset of microbial fermentation is a complex process that is influenced by a number of factors, including the type of juice, the temperature, and the presence of microorganisms. However, the insignificant difference in the brix value of karvand juice suggests that the sample was not fermented before analysis (Cheng *et al.,* 2007).

3.1.3 Cloud value:

The cloud value of juice is a critical quality parameter that affects the mouthfeel, shelf life, and consumer acceptance of juice. Cloud loss is a major problem in juice processing, as it can lead to a decrease in the quality of the juice. The present study investigated the effect of bath thermosonication on the cloud value of karvand juice.

The results showed that bath thermosonication significantly increased the cloud value of karvand juice. The present study demonstrates that there was a significant increase in cloud value from 0.163 of untreated karvand juice to 0.202 at 30 °C, 0.208 at 50°C, and 0.404 at 70°C absorbance units due to bath thermosonication (Table 2). Seshadri *et al.*, [2003] proposed a hypothesis that the application of ultrasound breaks the linear pectin molecule, reducing its molecular weight and resulting in weaker networks. This was attributed to the high-pressure gradient generated during cavitation, which leads to the breakdown of macromolecules into smaller counterparts. This breakdown of macromolecules results in the formation of a weaker network, which is less likely to precipitate and cause cloud loss [Gao and Rupasinghe, 2012]

The study also found that the increase in cloud value was more pronounced at higher temperatures. This is because higher temperatures lead to the formation of more bubbles, which in turn leads to more cavitation and a greater breakdown of macromolecules.

The findings of this study suggest that bath thermosonication is a promising method for improving the cloud value of karvand juice. This could lead to the development of new and improved juice products with a longer shelf life and better consumer acceptance.

Table 2: Effect sonication on pH, Titratable acidity, °Brix and Cloud Values of karvand juice

Sr.No.	Sample	pH **	Titratable acidity %	°Brix**	Cloud value**
1	Control	3.0±0.10	0.064	10±0.00	0.163±0.004
2	30°C	3.0±0.09	0.064	10±0.05	0.202±0.003
3	50°C	3.0±0.03	0.064	10±0.05	0.208±0.003
4	70°C	3.0±0.04	0.064	10±0.05	0.404±0.016

*****Means with the same letter do not show significant differences at P = 0.05***

3.1.4 Total antioxidant assay:

Antioxidants are important for human health because they help to protect the body from damage caused by free radicals. Free radicals are unstable molecules that can damage cells and DNA. Antioxidants can neutralize free radicals, preventing them from causing damage [Tungmunnithum *et al.*, 2018].

In the present study, it is observed that there is a significant increase in antioxidants content in karvand juice. Sonication treatments performed at 30°C, 50°C, and 70°C showed 10, 40, and 18 % increase in antioxidant content, respectively. The study mentioned found that sonication treatment can significantly increase the antioxidant content of karvand juice. This is because sonication can break down the cell walls of the karvand fruit juice, releasing the antioxidants that are trapped inside the cells. The increase in antioxidant content was more pronounced at higher temperatures, suggesting that sonication is more effective at higher temperatures.

The study also found that the increase in antioxidant content was sustained for up to 7 days after sonication. This suggests that sonication is a promising method for improving the antioxidant content of karvand juice without affecting its shelf life. Henceforth, the study provides evidence that sonication can be used to improve the antioxidant content of karvand juice. This could lead to the development of new and improved karvand juice products with potential health benefits.

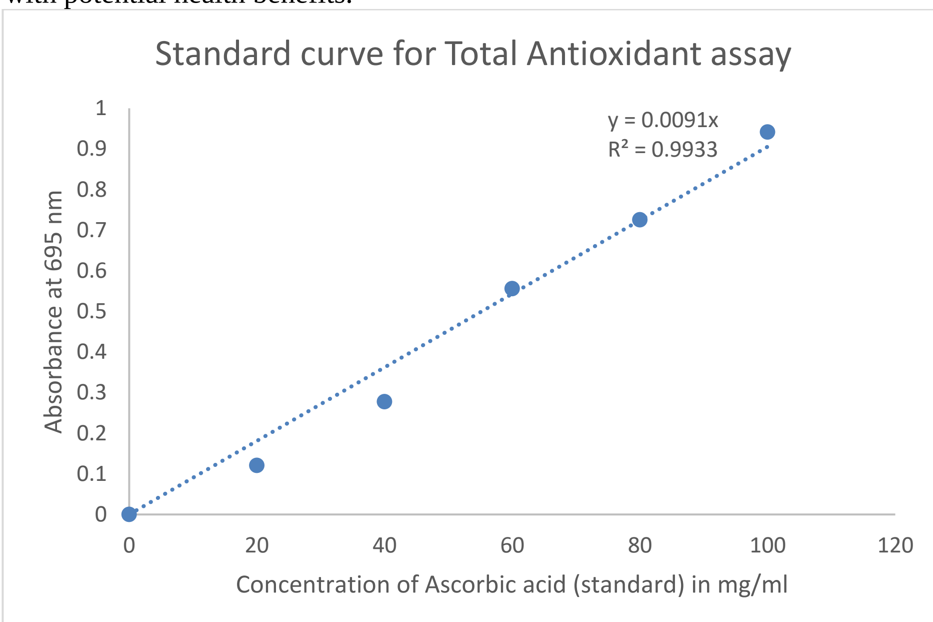

Figure 1: Standard Curve used for estimation of total antioxidant content

3.1.5 Total Phenolic and Total Flavonoids Content:

Phenolic compounds are antioxidants that have been shown to have health benefits. In this study, total phenolic has been found to be increased significantly in bath thermosonicated samples as compared to the control one. The total phenolic content in the control untreated sample was found to be 19.09 mg GAE/g (Table 3). Bath thermosonication at 30°C, 50°C, and 70 °C has been found to increase this total phenolic content up to 14.3, 18.6, and 17.4 mg GAE/g respectively.

Total flavonoid content in the Karvanda has shown a similar increase. Control unprocessed Karvanda juice was found to have 0.306 mg/g quercetin equivalents. Bath thermosonication at 30°C was found to have no significance on this flavonoid content and was found to be 0.405 mg/g quercetin equivalents. Bath thermosonication at 50 and 70°C was found to increase flavonoid content up to 0.247 and 0.530.

The results of this study are consistent with previous research on kasturi lime (Bhat *et al.*, 2011) and apple juice (Abid *et al.*, 2013). The increase in total phenolic content is likely due to the disruption of the cell wall by cavitation, which releases chemically bound phenolic compounds in the juice. The implosion of bubbles during sonication also generates hydroxyl radicals that can attach to the aromatic rings of phenolic compounds, further increasing their availability in the juice. Ashok *et al.*, (2008) have shown that the addition of hydroxyl groups to the ortho- or para-position of phenolic compounds increases their antioxidant activity. The study found that the use of ultrasound to process fruit juice can increase the number of phenolic compounds in the juice. The increase in phenolic compounds is likely due to the disruption of the cell wall by ultrasound, which releases these compounds from the cells. The implosion of bubbles during ultrasound also generates hydroxyl radicals, which can further increase the availability of phenolic compounds in the juice.

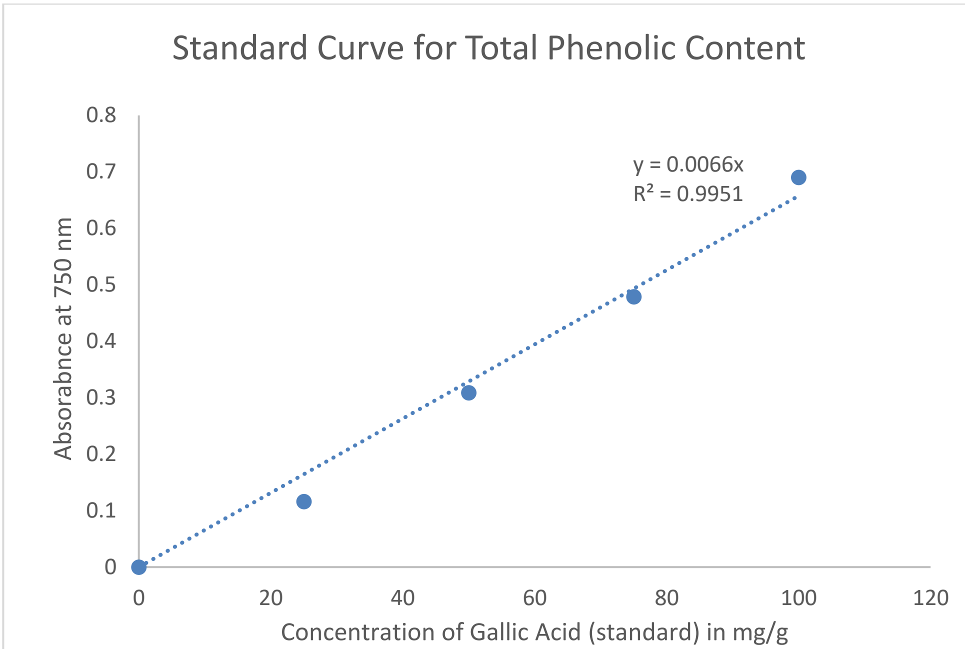

Figure 2: Standard Curve used for estimation of total phenolic content

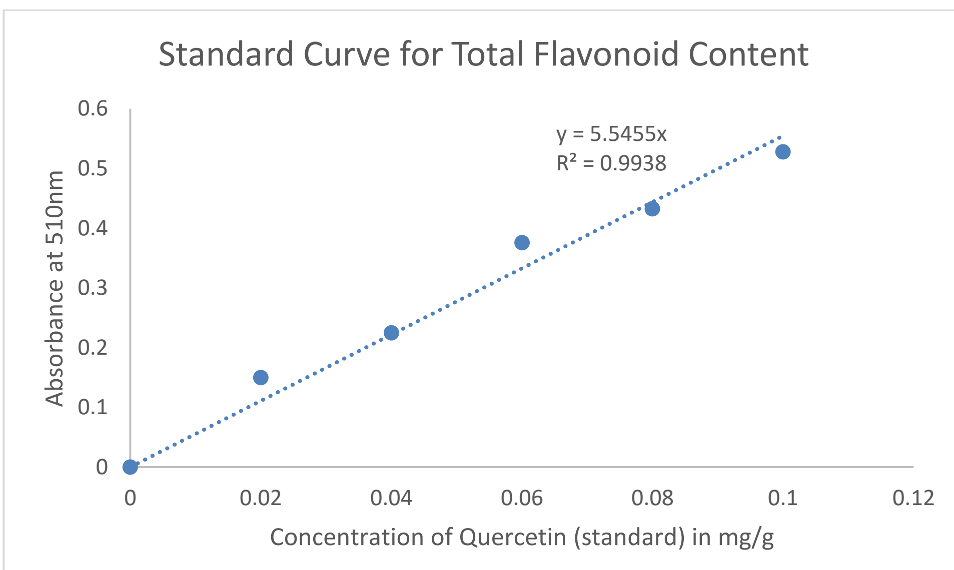

Figure 3: Standard Curve used for estimation of total flavonoid content

Table 3: Effect of sonication on antioxidants Total Phenolics, and Flavonoids

S.No.	Sample Treatment	Antioxidants (mg/ml)	Total Phenolics (mg/ml)	Flavonoid (µg/g quercetin equiv.) **
1	Control	12.2	14.4	0.3
2	30°C	13.3	18.6	0.4
3	50°C	17.0	17.4	0.2
4	70°C	14.7	19.1	0.5

CONCLUSION

The results of this study showed that sonication treatment was effective in retaining and enhancing the phenolic content and flavonoid content of the processed juice. Bioactive compounds, such as antioxidants, were also found to be increased in the processed juice. This suggests that sonication technology can be used to improve the quality of processed juices.

The study also found that bath thermosonication technology was able to retain the bioactive components in the processed juices, making it a promising novel processing method that overcomes the shortcomings of traditional processing technologies.

The present work suggests that future research should focus on optimizing the parameters of sonication treatment, such as frequency, temperature, and time, in combination with other physical food processing methods. They also believe that sonication treatment should be further investigated and compared to other methods, such as ultraviolet radiation, pulsed electric field, and modified atmospheric packaging.

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