Development of an Ultrasonic-Assisted Extraction Technique for the Extraction of Natural Coloring Substance Chlorophyll from Leaves of *Carica papaya*

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Abstract: Beautiful green leaves of Papaya are the rich source of Chlorophyll. Green color of chlorophyll has been used for a very long time as a natural colorant. *Carica papaya* has been considered as a good example and reasonable source of natural phytochemicals, which makes it suitable to color the food items and beverages. The aim of the present investigation is to develop the process of ultrasonic extraction in combination with solid phase extraction (SPE) to extract out chlorophyll with high yield as well as high degree of clarity. Newly customized ultrasonic-assisted extraction technique for the extraction of chlorophyll from *Carica papaya* leaves is optimized by taking different parameters like time, temperature, solvents concentrations, and raw material under consideration. Furthermore, the extract was purified by means of SPE and examined by using UV-Vis spectrophotometer. The highest yield of chlorophyll (dye) extract was found as 40% in solvent solution having 80 mL of ethanol and 20 mL of water with 5 minutes of extraction time, 35°C of temperature, and 1 grams of raw material in the sonication bath. Furthermore, the SPE purified sample was characterized by means of the UV-Vis spectrophotometer and here the total chlorophyll content was 34 mg/g, including chlorophyll a with a concentration of 14.1246 mg/g and chlorophyll b with concentration of 19.845 mg/g respectively. Consequently, sonication method can be suggested as a good method to get better concentration of chlorophyll.

Key words: *Carica papaya*, chlorophyll, solid phase extraction technique, UV-Vis spectrophotometer

1 Introduction

Papaya, scientifically known as *Carica papaya* belongs to family Caricaceae. It is a herbaceous tender plant with a strong stem. The whole plant (fruits, leaves, seeds, roots, juice, latex, and bark) is used for its nutritional, medicinal, and various other purposes. It is comprised of various phytochemicals including caffeic-acid, myricetin, quercetin, α-tocopherol, papain, benzyl iso-thiocyanate, kaempferol and rutin, that demonstrates its noteworthy antioxidant properties. It possesses antimicrobial, antihelmintic, and antioxidant properties. Extract of *Carica papaya* leaf has exceptional medicinal properties such as antibacterial, antiviral, antitumor, hypoglycemic, and anti-inflammatory activity. Also several *in vitro* and *in vivo* studies have verified that extracts of papaya leaf possesses following medicinal properties including antioxidant, anti-cancer, anti-dengue, antiplasmodial, anti-bacterial, hepato-protection and anti-inflammatory. *Carica papaya* is an ample and reasonable source of natural photochemical including chlorophyll. Previously reported research demonstrated that *Carica papaya* leaves have more amount of chlorophyll compared to other vegetables involving spinach, kale leaf and mustard greens. Due to outstanding nutritive profile and good concentration of chlorophyll (green colorant) in papaya leaves, its extraction with a suitable method has become essential requirement of today. However, with the developments, they have been replaced by the latest techniques having more extraction efficiency and the tendency to separate selective bioactive components to meet the growing demands of market.

Food color is a food additive which is not generally consumed as a food, but added to a consumable to give or restore color. The food color can be derived from any natural source or can be generated as artificial dyes. Natural sources are the best to produce tints due to their nutritive value. They not only fulfill the requirement of
uniqueness but also enrich the food with nourishing constituents\textsuperscript{9}. There is an immense increase towards the toxic results that are arising by the use of several additives in food and among them one of the very dangerous are Azo dye\textsuperscript{10}. Dyes are the common part of various industries as coloring agents. The discharge of dyes and particularly artificial dyes in wastewater, leads to severe environmental problems, which has become a public health concern\textsuperscript{11}. Replacement of synthetic colors by natural substitutes is attaining attention in the United States, European Union, and in further divisions of the world. Food and beverage productions have been fueled by customers increasing demand for natural food colors. Along with that they are also geared up due to few scientific reports on the possible danger of artificial food colorants\textsuperscript{12}.

Recently, the Green extraction methods including Supercritical fluid extraction, Ultrasound assisted extraction, microwave assisted extraction, pressurized liquid extraction, and pressurized hot water extractions are being used as alternatives of conventional extraction method\textsuperscript{13}. Due of poor extraction yield along with long time degradation of active components and less fastness properties the conventional methods of extraction are losing their significance. Now modern techniques are making prominent progress such as gamma radiation\textsuperscript{14}. The developed Ultrasound-assisted extraction technique has also been used in combination with the microwave extraction technique known as Ultrasound-Microwave-Assisted (UMA) extraction\textsuperscript{15}. For extraction purpose, an ultrasonic bath is preferable as it comprises of an ultrasonic sounder and piezoelectric transducer. This technique shows the best performance at lower temperature to prevent the damage to thermally sensitive bioactive components\textsuperscript{16}. Ultrasonic baths has several advantages like easy operation and it is economically suitable as well. On the other hand, ultrasonic probe promotes the mass transfer. It also increases the destruction of the sample because of the degradation of probe. However, it is importantly notable that it increases the temperature of the sample sharply\textsuperscript{17}. Generally, ultrasonic probe is built with layer structure that may contain wear plates, delay lines, acoustic lenses, electrodes, piezoelectric layers, matching layers and backing block\textsuperscript{18}. Consequently, the developed process of ultrasonic extraction combined with solid phase extraction was established to accomplish the extraction of chlorophyll with high yield as well as high degree of clarity. The following method is not only commercially advantageous and efficient but also contains health benefits. Resultantly, it is suggested to be a vigorous method to extract natural dye chlorophyll from the leaves of Carica papaya. This newly developed method has a good potential to be used on commercial scale for the efficient extraction of chlorophyll which has positive health attributes being a natural food colorant.

2 Materials and Methods
2.1 Raw material
Leaves of Carica papaya were collected from a local nursery of Pattoki. They were placed in a large tray and kept under shade for more than a week to dry, to prepare them for extraction.

2.2 Optimization of extraction condition
Ultrasound-assisted extraction was optimized by varying selected parameters such as solvent ratio, time, temperature and contact time. Solvent ratios were optimized with selected concentration ratios of ethyl alcohol and water (ethyl alcohol:water; 1:4, 2:3, 3:2 and 4:1 respectively). Sonication time was optimized by studying the extraction efficiency at selected time durations such as 5 minutes, 10 minutes, 15 minutes, and 20 minutes correspondingly. Extraction efficiency was further improved by varying the temperature of the sonication bath i.e, Separation was conducted by changing the temperature in the sonication bath as well. It i.e, 35°C, 40°C 45°C and 50°C while keeping the other parameters constant. During the extraction process, solutions were prepared by adding different amounts of papaya leaf powder such as 0.4 grams, 0.6 grams, 0.8 grams, and 1 gram, respectively\textsuperscript{18,19}.

2.3 Ultrasound-assisted extraction
Four samples were prepared in conical flasks having different concentrations of ethanol:water and 1 gram of Carica papaya leaf powder. The flasks were placed in the sonication bath for 5 minutes at 35°C. Sonicated solutions were then subjected to filtration and then further filtrate was placed in a hot water bath at a temperature between 80 to 90°C to remove the solvent and the yield was calculated. In the next step, the solvent concentration ratio providing the best yield was taken while the parameter of time was changed from 5 minutes to 10, 15, and finally 20. Again, the same process was repeated and the time resulting best yield was further taken to the next step. Identified concentrations of solvent along with known time that are giving better yield were taken into account while the parameter of temperature was changed now. The temperature giving the best yield was processed further. Finally, all above parameters providing the best yield were kept constant and different amounts of leaf powder were taken\textsuperscript{19}. Again after sonication the solution was filtered through a vacuum filtration pump and placed in a hot water bath for solvent evaporation, the yield was then calculated\textsuperscript{20}.

2.4 Solid phase extraction (SPE)
Conditioning of Cartridge was done with a solvent solution of ethanol and distilled water which was designed as 1:1. Now 5 mL of solvent was poured into the cartridge in two consecutive terms by keeping the pipette inclined to the side of the wall. Liquid should’n reaches the level of
meniscus because it will run dry with the sorbent. During loading, 5 mL of loading solution (extract) was added into the cartridge in two terms. A layer of the desired components started to appear in the sorbent. The sorbent was then washed to remove the impurities from it in the washing stage. Again, ethanol and deionized water solution 1:1 were used in a quantity of 5 mL for two consecutive turns. Now the beaker that was containing the filtrate was removed and another different solution was used in this stage of elution which contained 75 mL of ethanol with 25 mL of deionized water. Following solution added, eluted the desired analyte out of the sorbent and was collected in the receiving vial.21, 22

2.5 UV-Vis spectroscopy
To calculate the content of chlorophyll a and b individually and the total chlorophyll content a+b, the following formula was used with a bit of modification.23

\[
\text{chl}_a = 12.7 \text{Abs}_{663} - 2.7 \text{Abs}_{644}
\]

\[
\text{chl}_b = 22.9 \text{Abs}_{644} - 4.7 \text{Abs}_{663}
\]

Where chl\(_a\) was the chlorophyll a concentration and chl\(_b\) was the concentration of chlorophyll b. Abs\(_{663}\) while Abs\(_{644}\) stands for the absorbance of the sample at the wavelength of 663 nm and 645 nm correspondingly. Chlorophyll\(_a+b\) are the total chlorophyll content present in sample.24

3 Results and Discussion
The extraction yield of dye by using ultrasonic-assisted extraction method with different concentrations of ethanol in distilled water at 35°C temperature and 5 minutes time was shown in Fig. 1. Since Chlorophyll was more readily soluble in ethanol than water because there is a possibility that it might contains comparatively less polar functional groups and ethanol is also comparatively less polar than water, hence the binding capacity of ethanol is more towards chlorophyll. Therefore it showed the maximum amount in 80% ethanol.25, 26 The increase in Fig. 1 was noted because of the growing concentration of ethanol, which has enhanced the efficiency of extraction method.27

The extracting yield of colorant by means of ultrasonic-assisted extraction technique at different time intervals of time from 5 to 20 minutes at 35°C temperature was shown in Fig. 2. It expressed that the decrease in the amount of yield of dye might be due to the destruction of important components in the papaya leaves with an increase in time.28 Hence at the beginning the maximum amount of dye had been extracted and consequently the extraction ability has been reduced with the passage of time.29, 30 Here time of 5 minutes was suitable to acquire better yield. Finally, the optimized conditions for the better extraction were with solvent system 80 mL:20 mL ethanol with distilled water concentration and 5 minutes of residence time in the sonication bath.

Effect of temperature on the extracted yield was determined and analyzed while the concentration of ethanol was 80% with 20% distilled water and the sonication time was kept at 5 minutes, meanwhile different temperatures of 35°C, 40°C, 45°C and 50°C were set in the sonication bath. Results are given in Fig. 3. As at higher temperature there were more chances of the destruction of substances resulting the decrease in extracting substance was observed.29 Consequently, the suitable temperature for extraction was of 35°C.31 Until now, the optimized conditions for better extraction results were 80 mL:20 mL ethanol with distilled water concentration and 5 minutes of residence time in the sonication bath at the temperature of 35°C.

Results given in Fig. 4 showed that among all the
amounts of Raw material (0.4 g, 0.6 g, 0.8 g, 1 g), the highest extraction yield was observed in 1 gram. Sivakumar et al. also reported in their work that the total amount of colorant was increased with the increase in quantity of raw material (Beetroot) used. Another condition of 0.6 gram of raw material was also examined which produced a good yield of 38 at 5-minute time and 35°C temperature in the sonication bath. Hence, the suitable amount of raw material was either 1 gram or 0.6 gram. Consequently, all above results were in agreement with Qadariyah et al. who observed the extraction of lawsone from Hena leaves by means of optimized ultrasound assisted extraction technique.

Chlorophyll Content was determined by observing the absorbance of the sample at 663 nm and 644 nm via UV-Vis spectrophotometer and placed into the equation for the estimation of chlorophyll a and b. Absorbance value at 663 nm was 0.151 while at 644 nm it was 0.129.

\[
\text{chl}_a = 12.7 \text{Abs}_{663} - 2.7 \text{Abs}_{644} = 1.5694
\]

Yield of chlorophyll a was further multiplied by the total amount of sample which was 9 mL. Consequently, the concentration of chlorophyll a was 14.1246 mg/g.

\[
\text{chl}_b = 22.9 \text{Abs}_{644} - 4.7 \text{Abs}_{663} = 2.2057
\]

Yield of chlorophyll b was further multiplied by the total amount of sample which was 9 mL. Subsequently, the concentration of chlorophyll b is 19.845 mg/g. In order to determine the total chlorophyll content, both quantities were added.

\[
(a + b) = 14.1246 + 19.845 = 33.99 \text{ mg/g}, \text{ which was approximately equals to 34 mg/g.}
\]

Maulana, E. et al. found the concentration of chlorophyll in Carica papaya leaf as 74.80 mg/L. Meanwhile, the content of chlorophyll extracted out and purified by means of SPE was equal to 34 mg/g. Rizali et al. observed that 100% of the solvent concentration carries 3.1294 mg/m³ chlorophyll. In comparison to that, this work showed the extraction yield of chlorophyll was higher as 34 mg/g.

4 Conclusion

Consequently, the maximum amount of extracted dye (0.40 g/g) was found with the optimized sonication condition of ethanol and water (80:20) respectively, containing 1 gram of raw material with time duration of 5 minutes at 35°C temperatures. Moreover, the sample was purified by means of Solid Phase Extraction (using silica gel as a sorbent) selectively separated out the chlorophyll from rest of the coloring substance i.e. xanthophyll and carotene. Later the extracted material was analyzed by UV-Vis spectrophotometer at wavelength of 644 nm and 663 nm, respectively, that gave the total amount of chlorophyll as 34 mg/g, which is high and purified yield as compared to previous work done. Hence, Sonication method was not only less time taking but also less energy taking, which provides high yield of extract even at lower temperature.

Conflicts of Interests

All authors have no conflict of interest.

References

1) Nath, R.; Dutta, M. Phytochemical and proximate analysis of papaya (Carica papaya) Leaves. Sch. J. Agri.c Vet. Sci. 3, 85-87 (2016).

2) Airaodion, A.I.; Ogbuagu, E.O.; Ekenjoku, J.A.; O-
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J. Oleo Sci. 70, (10) 1367-1372 (2021)

3) Kumar, N.S.; Devi, P.S.S. The surprising health benefits of papaya seeds. *J. Pharmacobio-Divers. Sci.* 5, 227-234 (2019).

4) Kong, Y.R.; Jong, Y.X.; Balakrishnan, M.; Bok, Z.K.; Weng, J.K.K.; Tay, K.C.; Khaw, K.Y. Beneficial role of Carica papaya extracts and phytochemicals on oxidative stress and related diseases: A mini review. *Biol. Med.* 10, 287 (2021).

5) Ajiboye, A.E.; Olawoyin, R.A. Antibacterial activities and phytochemical screening of crude extract of Carica papaya leaf against selected pathogens. *Glob. J. Pure Appl. Math.* 26, 165-170 (2020).

6) Singh, S.P.; Kumar, S.; Mathan, S.V.; Tomar, M.S.; Singh, R.K.; Verma, P.K.; Acharya, A. Therapeutic application of Carica papaya leaf extract in the management of human diseases. *DARU J. Pharm. Sci.* 28, 1-10 (2020).

7) Nugroho, A.; Heryani, H.; Choi, J.S.; Park, H.J. Identification and quantification of flavonoids in Carica papaya leaf and peroxynitrite-scavenging activity. *Asian Pac. J. Trop. Biomed.* 7, 208-213 (2017).

8) Mulgund, N.T. Dengue virus disease: Current updates. *Int. J. Sci. Res. Innov. Stud.* 4, 36-50 (2017).

9) Bajwa, B.; Mazhar, M.S.; Bashir, M.K.; Honey, S.F. Environmental, economic and social impact of biological control interventions in papaya farming in Sindh, Pakistan. *Pak. J. Life Sci. Soc.* 16, 27-34 (2018).

10) Oplatowska-Stachowiak, M.; Elliott, C.T. Food colors: Existing and emerging food safety concerns. *Crit. Rev. Food Sci. Nutr.* 57, 524-548 (2017).

11) Lellis, B.; Fávaro-Polonio, C.Z.; Pamphile, J.A.; Polonio, J.C. Effects of textile dyes on health and the environment and bioremediation potential of living organisms. *Biotechnol. Res. Innov.* 3, 275-290 (2019).

12) Vinha, A.F.; Rodrigues, F.; Nunes, M.A.; Oliveira, M.B.P. in *Natural Pigments and Colorants in Foods and Beverages*, 1st ed. Woodhead Publishing Ltd., Cambridge, pp. 363-391 (2018).

13) Ameer, K.; Shahbaz, H.M.; Kwon, J.H. Green extraction methods for polyphenols from plant matrices and their byproduct. *Compr. Rev. Food Sci. Food Saf.* 16, 295-315 (2017).

14) Zia, K.M.; Adeel, S.; Aslam, H.; Khosa, M.K.; Zuber, M. Influence of ultrasonic radiation on extraction and green dying of mordanted cotton using neem bark extract. *J. Ind. Eng. Chem.* 77, 317-322 (2019).

15) Wizi, J.; Wang, L.; Hou, X.; Tao, Y.; Ma, B.; Yang, Y. Ultrasound-microwave assisted extraction of natural colorants from sorghum husk with different solvents. *Ind. Crop Prod.* 120, 203-213 (2018).

16) Chemat, F.; Rombaut, N.; Sicaire, A.-G.; Meullemiestre, A.; Fabiano-Tixier, A.-S.; Abert-Vian, M. Ultrasonic assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. *Ultrason. Sonochem.* 34, 540-560 (2017).

17) Svilainis, L.; Kybartas, D.; Aleksandrovas, A.; Alvarez-Arenas, T.E.G. High frequency focused imaging for ultrasonic probe integrity inspection. *NDT and E Int.* 116, 1-15 (2020).

18) Qadariyah, L.; Azizah, N.; Syafa‘atullah, A.Q.; Bhuanu, D.S.; Mahfud, M. The extraction of natural dyes from Henna leaves (*Lawsonia inermis L.*) by ultrasound-assisted method. *Matier. Sci. Eng. C 543*, 1-7 (2019).

19) Nutter, J.; Fernandez, M.V.; Jagus, R.J.; Agüero, M.V. Development of an aqueous ultrasound-assisted extraction process of bioactive compounds from beet leaves: A proposal for reducing losses and increasing biomass utilization. *J. Sci. Food Agric.* 101, 1989-1997 (2021).

20) Saberian, H.; Hosseini, F.; Bolourian, S. The effect of ultrasound method on the extraction of chlorophyll from mulberry leaves. *Innov. Food Sci. Emerg. Technol.* 47, 67-76 (2017).

21) Moghimi, A.; Yari, M. Review of procedures involving separation and solid phase extraction for the determination of cadmium using spectrometric techniques. *Chem. Rev.* 1, 1-18 (2019).

22) Kraševč, I.; Prosen, H. Solid-phase extraction of polar benzotriazoles as environmental pollutants: A review. *Molecules* 23, 1-14 (2018).

23) Özcərəberoğlu, N.; Kahramanoğlu, İ. Mathematical models for the estimation of leaf chlorophyll content based on RGB colours of contact imaging with smartphones: A pomegranate example. *Folia Hortic.* 32, 57-67 (2020).

24) Makarska-Bialokoz, M.; Kaczor, A. A. Computational analysis of chlorophyll structure and UV-Vis spectra: A student research project on the spectroscopy of natural complexes. *Spectrosc. Lett.* 47, 147-152 (2014).

25) Zhang, Z.H.; Peng, H.; Woo, M.W.; Zeng, X.A.; Brennan, M.; Brennan, C.S. Preparation and characterization of whey protein isolate-chlorophyll microcapsules by spray drying: Effect of WIPI ratios on the physicochemical and antioxidant properties. *J. Food Eng.* 267, 1-8 (2020).

26) Rubah, S.; Bahadur, S.; Hanif, U.; Durrani, A.I.; Sadiqa, A.; Shafiique, S.; Iqbal, S. Phytochemical and antimicrobial investigation of methanolic extract/fraction of *Ocimum basilicum* L. *Biocatal. Agric. Biotechnol.* 31, 1-10 (2021).

27) Kunene, P.N.; Mahlambi, P.N. Optimization and application of ultrasonic extraction and Soxhlet extraction
followed by solid phase extraction for the determination of triazine pesticides in soil and sediment. *J. Environ. Chem. Eng.* 8, 1-9 (2020).

28) Mahindrakar, K.V.; Rathod, V.K. Ultrasonic assisted aqueous extraction of catechin and gallic acid from Syzygium cumini seed kernel and evaluation of total phenolic, flavonoid contents and antioxidant activity. *Chem. Eng. Process* 149, 1-7 (2020).

29) Ying, Z.; Han, X.; Li, J. Ultrasound-assisted extraction of polysaccharides from mulberry leaves. *Food Chem.* 127, 1273-1279 (2011).

30) Meng, Z.; Zhao, J.; Duan, H.; Guan, Y.; Zhao, L. Green and efficient extraction of four bioactive flavonoids from Pollen Typhae by ultrasound-assisted deep eutectic solvents extraction. *J. Pharm. Biomed. Anal.* 161, 246-253 (2018).

31) Hu, Y.; Kwan, T.H.; Daoud, W.A.; Lin, C.S.K. Continuous ultrasonic-mediated solvent extraction of lactic acid from fermentation broths. *J. Clean. Prod.* 145, 142-150 (2017).

32) Sivakumar, V.; Anna, J.L.; Vijayeeswarri, J.; Swaminathan, G. Ultrasound assisted enhancement in natural dye extraction from beetroot for industrial applications and natural dyeing of leather. *Ultrason. Sonochem.* 16, 782-789 (2009).

33) Maulana, E.; Pramono, S.H.; Fanditya, D.; Julius, M. Effect of chlorophyll concentration variations from extract of papaya leaves on dye-sensitized solar cell. *Int. J. Electr. Comput. Eng.* 9, 49-52 (2015).

34) Rizali, D.; Suryanto, H.; Sukarni, S. The effect of chlorophyll concentration from papaya leaves on the performance of dye sensitized solar cell. *J. Mech. Sci. Technol.* 3, 59-69 (2020).

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