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COMPARATIVE SEQUENTIAL SOLVENT EXTRACTION OF PHYTOCHEMICALS FROM RIPE, RIPENING AND UNRIPE *CARISSA CARANDAS* FRUIT EXTRACTS AND THEIR ANTIOXIDANT INVESTIGATION

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Abstract

Present study was structured to explore the full potential of *Carissa carandas* fruits whose potential is unidentified in Pakistan. Comparative study of unripe white, ripening pinkish and fully ripe reddish-purple fruit was commenced with sequential solvent extraction (10g/100 mL) by following aqueous (aq.), methanol, ethyl acetate, chloroform and n- hexane to investigate maximum phytochemicals. The results showed progressive extraction in methanol and aq. extracts as both were observed rich in phytochemicals. The high total antioxidant contents were found 65 and 105 mg/g in aq. and methanol extract of ripe fruit respectively as compared to unripe and ripening extract fractions. Percentage of radical scavenging activity (%RSA) was found maximum of 76 and 74% for 50 µL aq. fractions of ripening and unripe fruits respectively while 100 µL aq. ripe fruit extract showed maximum 44% RSA. Whereas 200 µL of ripe fruit methanol extract showed 83% high RSA while 250 µL of ripening fruit exhibited 78% RSA. The maximum 45% RSA was obtained with 250 µL of unripe fruit methanol extract. Significantly high 88% of hydroxyl radical scavenging activity (%HRSA) was observed with 200 µL of ripening fruit aq. extract, 70% for ripe and 43% for unripe fruit was obtained with 50 µL extract dilutions. Highest %HRSA was calculated for 100 µL ripening fruit methanol extract although 69 and 66 % was estimated for 50 µL of ripe and un- ripe fruit extracts. Thus, ripe fruit showed best results in phytochemical analysis having good antioxidant content and highest antioxidant activity.

Key words: Phytochemicals; *Carrisa carandas*; Antioxidant; Ripe fruit; Radical scavenging.

Introduction

With the improvement in quality of life, global trend towards medicines has been changed from chemically synthesized to natural source of medicines. Plants in all cultures play a noteworthy role in this regard, maintaining health and fraught to cure diseases (Singh & Uppal, 2015). Therefore, in this growing era, a demand of remedial plants has been increased. In therapeutic world, among other beneficial plants *Carissa carandas* is unexplored plant; an enormous research could be made to explore its remedial characteristics so that the coming generation can procure this paragon of nature (Wani *et al*., 2013). In Pakistan, Herbal Medicine Department remained unaware with the beneficial aspects of this plant and gave no promotion to its cultivation (Arif *et al*., 2016). Whereas in tropical and subtropical regions of the world; it is cultivated as various parts of this plant is used in traditional medicines (Bhaargavi *et al*., 2014).

Carissa carandas belongs to Apocynaceae family, is an evergreen shrub with 2 - 4 meters height. The plant has rhombus and conical leaves, white flowers and berry like fruits, arranged in clusters of 3 - 10 fruits having several seeds (Sutar & Sawant, 2012). Young unripe fruit is white to pinkish white; at ripening is entirely pink while ripe fruit is red to dark purple. The red purple juice of ripe fruit is very popular as it is rich in iron and vitamin C, therefore can be used in treatment of anemia and as antiscorbutic (Singh & Uppal, 2015). As ripe fruit is rich in pectin, acids, micro and macro nutrients, it is also used in the preparations of jams, jellies, curries, condiments and beverages in countries where it is cultivated plant as crop (Maheshwari *et al.*, 2012). The fruit flesh and its juice are a rich source of contents due to evaluate its antioxidant, antimicrobial, anti-diabetic, anti-inflammatory and anti-cancerous potential (Naing, 2011). One more biological aspect is its antioxidant potential against harmful oxidants that cause skin cancer (Salunke & Ghate, 2013). It also possesses reducing scavenging activity towards superoxide anions, hydroxyl radicals and H_2O_2 (Khushboo *et al*., 2016).

Present study was conducted to explore phytochemical characteristics of this nature gifted shrub, as most of people in Pakistan are still not aware of this treasure. Each part of this plant including fruit, leaves, roots, shoots, and seeds has its own specialty (Aslam *et al*., 2012). This research covered phytochemical analysis in the different forms of its fruit, their sequential extraction from polar to non-polar and their antioxidant potential in optimum solvent. Fully ripe, ripening and unripe fruits were taken under observation to find out which is additional expedient.

Materials and Methods

The fully flourished plant material was procured from a local nursery near main Gulberg and identification of plant was done by Dr. Zaheer Uddin khan, distinguish Professor of Botany Govt. College University, Lahore, Pakistan with voucher #GC. Herb. Bot. 3448. All the fruits were removed and thoroughly washed with distilled water, dried and segregated on the basis of unripe, ripening and ripe fruit. The three types of fruits were kept in separate polythene bags and refrigerated at 4^{o}C for further phytochemical investigations.

Sequential phytochemicals cold extraction method: To find the best, cheap and safe solvent for phytochemicals extraction from three different grades of fruit; this method was carried out from polar to non-polar solvent; decreasing order of such as water, methanol, ethyl acetate, chloroform and n- hexane (Sawant & Godghate, 2013). The three types of fruits (10g each without seeds) were homogenized with 100 mL of doubled distilled water at room temperature (25ºC) and centrifuged at 4000 rpm to get the pellet of residue. The pellet was soaked in 50 mL methanol for cold extraction for three days, centrifuged to get pellet at 4000 rpm and supernatant layer of methanol extract was kept for further analysis. The same process of sequential cold extraction was carried out to next solvents of decreasing order of polarity. All obtained extracts were stored in vials for further phytochemical and antioxidant investigations.

Phytochemical investigations: Different reported tests (Dhruti *et al*., 2016) were used to determine the basic important bioactive compounds in various solvent layers of three grades; unripe, ripening and ripe fruit of *Carissa carandas*. All the chemicals used were of analytical grade to prepare reagents for qualitative analysis of carbohydrates, phenols, flavonoids, anthocyanins, tanins, *Alkaloids*, steroid, emodins, coumarin, diterpenes, phytosterol, *Cardial Glycosides*, protein, amino acids and saponins.

Antioxidant activity

Phosphomolybdenum assay: The antioxidant activity of three stages extracts were measured by phosphomolybdenum complex formation by following the method of Preito (1999) with some modifications; the reagent was prepared by adding 2.94 mL of conc. sulfuric acid to 0.245 gm ammonium molybdate and 0.18 gm of sodium triphosphate; it was dissolved in 20 mL of distilled water and finally 50 mL volume was made with distilled water.

From each extract sample, 1mL was taken in dry test tubes having 0.5 mL of DMSO and 1 mL of above reagent was added in it. All the test tubes were sealed and incubated at 95ºC for 90 minutes. Then test samples were cooled to room temperature (25ºC) and 1 mL of DMSO was added and absorbance was measured at 695nm by UV- spectrophotometer. Ascorbic acid (0.06g/10mL DMSO) was used as standard. Antioxidant activity was uttered as the number of equivalents of ascorbic acid and was calculated by following equation:

$$\mathbf{A} = \frac{c \times V}{m}$$

where A is total antioxidant contents of fruit extract in mg/g while c is the concentration of ascorbic acid established from calibration curve mg/mL while V is equal to the volume of extract and m is weight of crude plant extract (Afizah *et al*., 2016). The phosphomolybdenum reduction potential (PRP) of the studied extract was given in percentage.

DPPH (2, 2- diphenyl -1-picryl hydrazide) assay: Radical scavenging activity of different extracts was determined by using DPPH assay according to Chang (2001). The decrease in absorption of the DPPH solution after the addition of an antioxidant was measured at 517nm. 0.1mM DPPH solution was prepared by dissolving 0.004g of DPPH in 100ml of ethanol. Different volumes of aqueous and Methanolic fruit extracts (50, 100, 150, 200 and 250µL) were diluted with 500µL of DPPH. The reaction mixture was incubated in dark condition at 30ºC for 20 min and absorbance was read at 517 nm with UV-visible spectrophotometer. 3mL of DPPH solution was taken as control and ethanol as reference. The % radical scavenging activity (RSA) of the fruit extract was calculated using the following formula,

$$\% \text{ RSA} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

where, Abs control and Abs sample is the absorbance of DPPH and absorbance of fruit extract (Duh 1998).

Hydroxyl radical scavenging assay: This assay was carried out to optimize different concentrations of aqueous and methanol fractions of all fruit grades. The reaction reagents were prepared by following Halliwell and Gutteridge (1989) with some modifications. Nash reagent was prepared by dissolving 7.5g ammonium acetate in 0.5 mL of glacial acetic acid and 0.2 mL of acetone in 100 mL of distilled water. Various concentrations of extract (50, 100, 150, 200 and 250 µL) were taken in clean dried test tubes in 1 mL iron- EDTA (0.13% ferrous ammonium sulfate (Mohr salt) and 0.26% EDTA) solution, 0.5 mL 0.018% EDTA, 0.5 mL of DMSO and 0.5 mL 0.22% ascorbic acid and incubated at 95ºC for 15 minutes. Then mixture was cooled at room temperature (25ºC); added 1mL of ice cold 0.75% TCA, 3 mL Nash reagent, incubated at room at 30ºC for 15 minutes and -absorbance was measured at 412 nm. The %HRSA is calculated by formula:

$$\% \text{ HRSA} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

where, HRSA is the Hydroxyl Radical Scavenging Activity, Abs control is the absorbance of control and Abs sample is absorbance of extracts.

Results and Discussion

Carissa carandas fruit is relatively new to market and needs promotion in national and international market (Arif *et al*., 2016) as this fruit has three prominent forms: unripe white, ripening pinkish and fully ripe reddish-purple fruit that are rich in vitamins, minerals and bioactive phytochemicals (Sawant & Godghate, 2013). To investigate various phytochemicals and their compatibility in a particular solvent; sequential solvent extraction (from polar to non- polar) was designed during this study. Comparative qualitative phytochemical investigation in three different stages of

Carissa carandas fruit were carried out prior to antioxidant analysis. For this purpose, 10 g of each fruit as shown in Fig. 1 was used for sequential solvent extraction of phytochemicals. Different reported reagents were used to determine famous phytochemicals that were actively played role in biomedicines. The comparative study of different phytochemicals in all fruit extract fractions are given in table.

After getting different extracts by sequential extraction, these extracts were subjected to qualitative analysis to find best phytochemicals extracting solvent. The comparative results of all three forms of *Carissa carandas* fruit are depicted in table 1 with following keys: strong presence (+++), medium presence (++), weak presence (+) and absence (-) of important phytochemicals. *Carissa carandas* showed progress in methanol and aqueous extracts as both were observed rich in various phytochemicals like steroids, alkaloids, flavonoids, carbohydrates, amino acid, coumarin, tannins, cardiac glycosides, emodins, phytosterols, saponins and phenolic compounds. While in $CHCl_3$ layer only steroids, carbohydrate, emodins, alkaloids, phytosterols, cardiac glycosides and diterpenes were present and rest of phytochemicals were absent. In ethyl acetate and n-hexane layer steroids, cardiac glycosides, phytosterols, alkaloids and proteins were present. Generally ripe fruit covered best action in phytochemical analysis. The presence of above mentioned important bioactive constituents impart medicinal characteristics to all the forms of this fruit (Kaunda & Zhang, 2017). Sedak *et al*., (2013) also investigated phytochemicals from ethanolic and n-hexane extract of *Carissa carandas* leaves and found results that are in line with present findings; having more phytochemicals in polar solvent as compared to non-polar. Naing (2011) also investigated presence of important phytochemicals in the root of *Carissa carandas* extract by using polar solvents that were also in agreement of present findings. Dhruti *et al*., (2016) also performed phytochemical analysis of *Alstonia scholaris* and revealed significant phytochemical presence in aqueous and methanolic extracts; these results are in line with present results as well. Behlil *et al*., (2019) performed qualitative analysis of methanolic extracts of *E. thyrsiflora*, *C. stocksii* and *G. macrantha* and found maximum phytochemicals in those extracts that was further in agreement of present findlings.

Antioxidant analysis: Food items are deteriorated by oxidation reactions and synthetic antioxidants like butylated hydroxytoluene and Butylated hydroxyanisole have serious health concerns with the use as food additives (Onyeulo *et al*., 2018). As more than 8000 various active biomolecules are investigated from fruits and vegetable origin that can be used as a better replacement of antioxidants than synthetic ones (Altemimi *et al*., 2017). Therefore, after completing comparative phytochemical study of all stages fruit; antioxidant analysis was performed by measuring total antioxidant content of all solvent extracts of three fruit stages. Phosphomolybdenum method was found very easy and approachable to calculate total antioxidant content in mg/g of fruits shown in Fig. 2. The basic principle of this method was reduction of phosphomolybdic acid by antioxidant present plant extracts and formation of blue complex by sodium sulfide as a representation of total antioxidant contents in that extract. Comparative analysis showed maximum antioxidant content 105 mg/g in methanol extract of ripe fruit whereas ripening and unripe fruit showed highest contents 102 and 98 mg/g respectively. The highest antioxidant contents found in aqueous and methanol extracts can be attributed with strong presence of phytochemicals in these extracts that are responsible to their antioxidant activity. Other sequential extracts (ethyl acetate, chloroform and n. hexane) also showed considerably low concentration of antioxidant contents. These results were also well-matched to phytochemical investigations (Hepsibah & Jothi, 2017) as most of phytochemicals were weakly present or absent in those extracts. As it was first time reported work of *Carissa carandas* different stages of fruit that give a complete comparative result of phytochemicals and antioxidant contents. The results illustrated that ripe fruit was rich in phytochemical and showed highest contents of antioxidants. For the extractions of such bioactive phytochemicals best suggested solvent is distilled water and methanol. Further with the comparative study of three stages; it was verified that maximum extraction of phytochemicals was resultant in aqueous and methanol. So, a large-scale phytochemical extraction these two solvents would be the best choice and can be used for biomedical applications. Further antioxidant activity of these two extracts was studied by measuring percentage of radical scavenging activity and hydroxyl scavenging activity to optimize concentration of extract.

Radical scavenging activity: The stable free radicals purple in color generated by DPPH activity were scavenged by bioactive molecules that was attributed due to its antioxidant activity. This turns the medium yellow in color and consequently decrease of absorbance was noticed at 517 nm that specify the scavenging potential of those fractions of plant extracts. Different dilutions (50, 100, 150, 200 and 250 µL) of aqueous and methanol extracts were taken to optimize conc. of extract showing maximum % RSA. The results explained %RSA for aqueous extracts of ripe, ripening and unripe fruit a shown in fig. 3 that 50 µL ripening and unripe fruit extract presented highest % RSA of 74 and 76 %; respectively whereas 44% was found maximum with 100 µL of ripe fruit extract. Ripening and unripe fruit extracts documented also good % RSA for other diluted fractions as well whereas ripe fruit extract showed decrease in %RSA with increase of fraction concentrations. However, in the comparative study of methanol extracts, high % RSA was 83 % with 200 µL ripe extract, 78% of ripening and 45% of un- ripe with 250 µL of methanol extract. A good % RSA was observed for all fractions of ripe and ripening fruit whereas less % RSA was obtained from unripe fruit extract as shown in Fig. 4.

Table 1. Comparative phytochemicals investigation of *Carissa carandas* fruits in three different stages.

Qualitative test	Un- ripe fruit extracts					Ripening fruit extracts					Ripe fruit extracts				
	Aq.	**MeOH**	**E.A**	**$CHCl_3$**	**n- Hex**	**Aq.**	**MeOH**	**E.A**	**$CHCl_3$**	**n- Hex**	**Aq.**	**MeOH**	**E.A**	**$CHCl_3$**	**n- Hex**
Steroids (Red color)	+++	++	-	+++	+++	+++	++	-	++++	+++	+++	++++	+++	++	+
Tannins															
Lead acetate (yellow color)	++	+++	-	-	-	++	++	+++	-	-	+++	++++	-	-	-
$FeCl_3$ test (Green color)	-	-	-	-	-	-	-	-	-	-	+++	++	-	-	-
Carbohydrates															
Molisch test (violet ring)	++++	+++	-	+++	++++	++++	++	-	+++	+++	+++	++++	-	+	+
Fehling test (Brick red)	-	++++	-	++++	-	-	+	-	++++	+	++	++	-	++++	-
Flavonoids															
Alkaline reagent (yellow ppt)	-	++++	-	-	-	+	+++	-	-	-	++++	++++	-	+	+
NH_4OH (yellow ppt)	++	++++	-	-	-	+++	++++	-	-	-	++++	++++	-	-	-
Zn salt test (red color)	-	-	-	-	-	++++	++++	-	-	-	+++	++++	-	-	-
Saponins (foam formation)	++++	-	-	-	-	++++	++	-	-	-	++++	+++	+	-	-
Emodins: treated with NH_4OH and Benzene (red color)	-	-	-	++++	-	-	-	-	+	-	++	-	-	+++	-
Coumarins: treated with 10% NaOH (yellow color)	++	++++	-	+	-	++	+++	-	-	-	++++	++++	-	-	-
Diterpenes: Copper acetate test (emrald green)	+++	+	++	+++	-	+	++	+++	-	-	+++	++++	+	-	-
Phytosterol: Salkowski test (golden red)	++++	+++	++	+++	++	+++	++	++	+++	++++	+++	++++	++	+++	+++
Cardial glycosides: Keller-Killani Test (brown color)	++++	+++	-	+++	+++	++++	++++	-	++	+++	++++	+++	++	+++	+++
Phenol															
Alcoholic $FeCl_3$(bluish black)	-	-	-	-	-	++	+++	+	-	-	+++	++++	-	-	-
Proteins															
Xanthoproteic test (yellow ppt)	+++	++	++	+	++	++++	+++	++++	+++	+++	+++	++++	+++	+	-
Biuret test (violet pink)	+	++	-	-	-	-	-	-	-	-	++	++++	-	-	-
Amino acids (violet color)	+	++	-	-	-	++	-	-	-	-	+	++++	-	-	-
Alkaloids															
Wagner test(orange color)	++++	++++	+++	-	+	++++	++++	++	+	+	++++	+++	++++	++++	+
Hager test (yellow color)	++++	++++	+	++	+	++++	++++	-	++	+	++++	++++	++	++	++
Anthocyanin (extracted treated with 2N HCl resulted violet after pink)	+++	+	-	-	-	+++	+	-	-	-	++++	++++	-	-	-

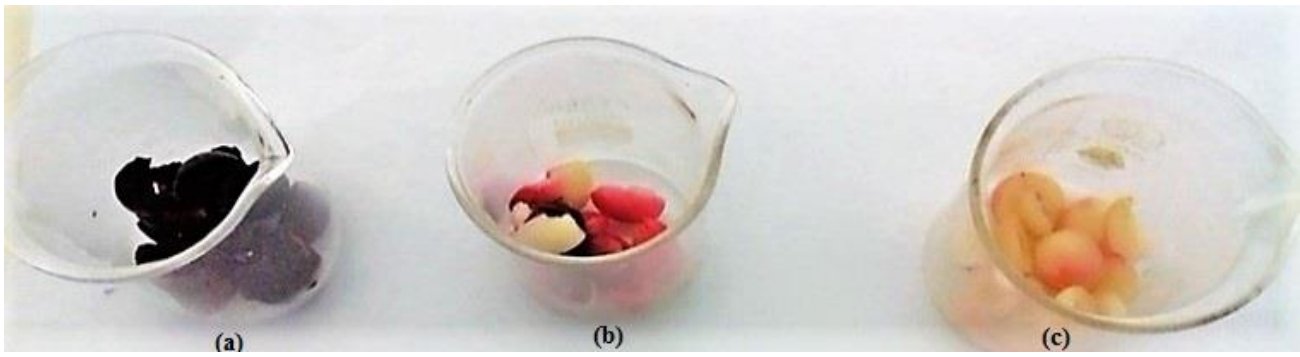

Fig. 1. Three different stages of *Carissa carandas* half cut without seed fruits for sequential solvent cold extraction (a) ripe (b) ripening (c) unripe.

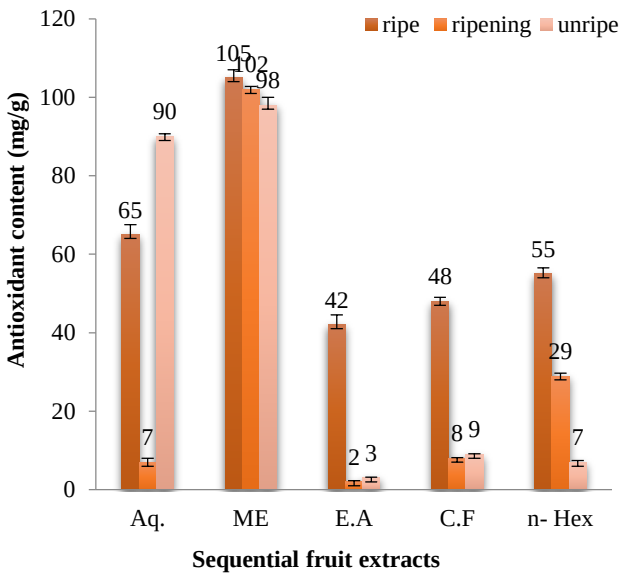

Fig. 2. Antioxidant contents (mg/g) found in fruit of *Carissa carandas* (three stages) sequential extracts; doubled distilled water (aq.), methanol (ME), ethyl acetate (E.A), chloroform (C.F) and n- hexane (n- Hex).

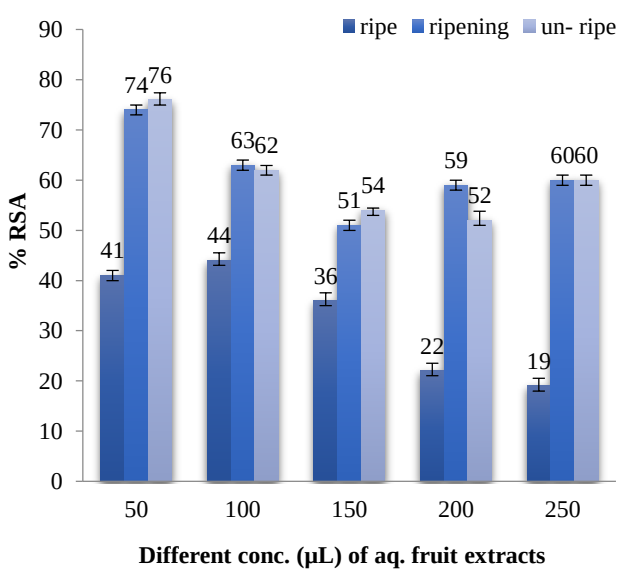

Fig. 3. % of Radical scavenging activity (%RSA) for different conc. of three stages fruit of *Carissa carandas* aqueous extract.

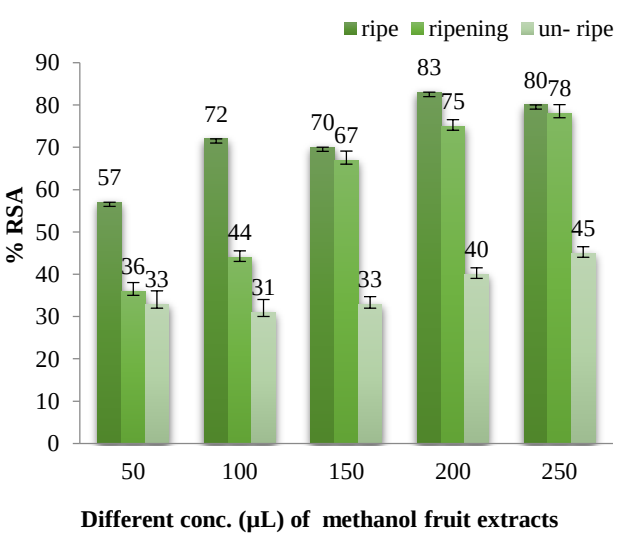

Fig. 4. % of Radical scavenging activity (%RSA) for different conc. of three stages fruit of *Carissa carandas* methanol extract.

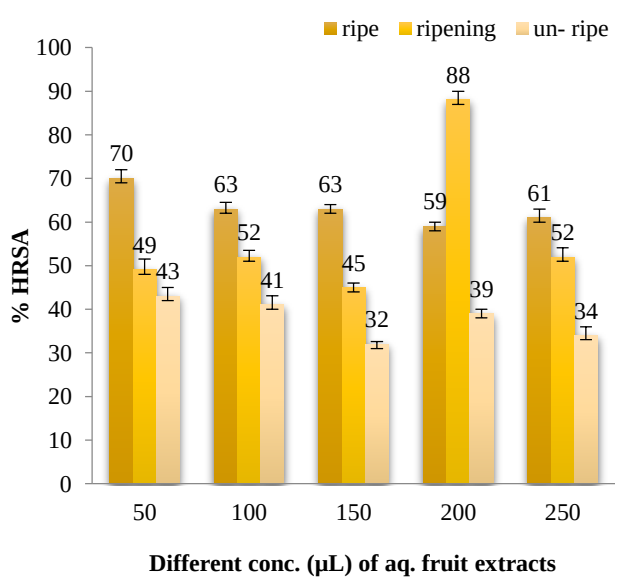

Fig. 5. % of Hydroxyl radical scavenging activity (%HRSA) for different conc. of three stages fruit of *Carissa carandas* aqueous extract.

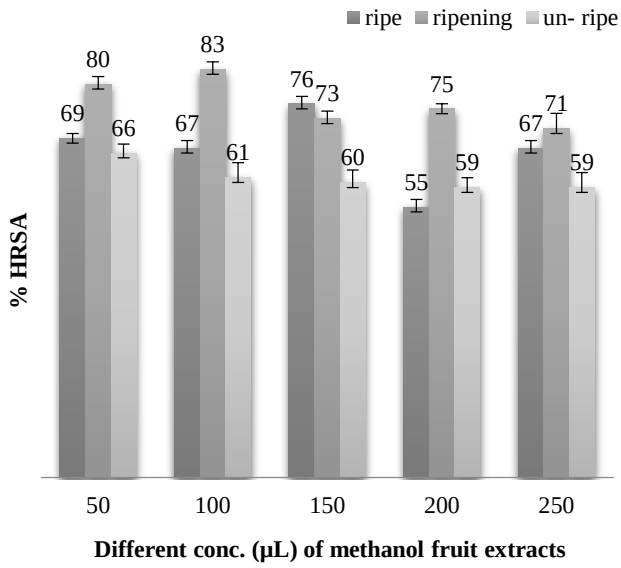

Fig. 6. Hydroxyl radical scavenging activity (% HRSA) for different conc. of three stages fruit of *Carissa carandas* methanol extract.

Hydroxyl radical scavenging activity: The antioxidant potential of plant extracts would be another application that can be used as scavenging for hydroxyl free radicals. These radicals cause damage to body cells like H_2O_2 generated in body cells is a potential cancer cause. This can be scavenged by potential plant extracts. In this method hydroxyl radical is generated when ascorbic acid-iron- EDTA interact with each other. The decrease of absorbance than control was attributed with the hydroxyl scavenging potential of extracts; Fig. 5 showed the comparison of the %HRSA results of each kind of fruit aqueous extract for various fractions. Maximum % HRSA of 88 % was observed for 200 µL ripening fruit extract from all the other fractions of ripe and unripe fruit aq. extracts although 50 µL of ripe and unripe fruit extract depicted 70% and 43% HRSA respectively. Whereas all the dilutions of methanol extracts showed maximum % HRSA as represented in Fig. 6 with maximum 83% with 100 µL dilutions of ripening fruit extract while 76% and 66% was observed for 150 and 50 µL of ripe and unripe fruit extracts. While comparing both aqueous and methanol extracts it was found that methanol extracts documented high trend of % HRSA that was in agreement with highest concentrations of bioactive molecules in methanol layer from all the other examined solvents.

Sarma *et al*., (2015) evaluated the antioxidant activity of Methanolic extract of *Carissa caradas* fruit by using free radical 1,1- diphenyl- 2- picrylhydrazyl (DPPH) and found IC50 value 27.45±0.43 µg/mL. Sueprasarn *et al*., (2017) investigated 1% HCl in 95% ethanol extract of Karonda for antioxidant activity by DPPH assay and found % of inhibition 78.28±0.12%. Ramakrishna *et al*., (2012) studied In vitro antioxidant activity of *Leucas linifolia* crude protein extract (CPLL) by following hydroxyl radical and DPPH scavenging activity. The maximum 78 and 63% inhibition was observed with 500µg/mL concentration of CPLL; IC_{50} value of 150 and 175 µg/mL was obtained for both above mentioned antioxidant activities respectively. Sadek *et al*., (2013) studied ethanolic and n- hexane leaves extract of *Carissa carandas* and found significant antioxidant activities as associated to standard antioxidants like ascorbic acid and BHT in DPPH assay; with IC_{50} of 1.292 and 1.824µg/mL for both extracts respectively. Afizah *et al*., (2016) examined the ethanolic and n- hexane extract of *Carissa carandas* fruit for antioxidant activities and found significant results as compared to synthetic antioxidant for DPPH assay; with IC_{50} 1.44 and 1.98µg/mL for both extracts respectively.

Conclusion

Thus, by comparative sequential solvent extraction showed that all three forms of *Carissa carandas* fruits; unripe, ripening and ripe were rich in bioactive phytochemicals that making medicinally important fruit. Moreover, aqueous and methanol extracts were found better solvents for large scale extraction of utmost phytochemicals. Red purplish fully ripe fruit exhibited maximum phytochemicals and better antioxidant activity compared to all the other stages of fruit. Consequently, ripe fruit of *Carissa carandas* presented promising sequel.

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