COMPARATIVE SEQUENTIAL SOLVENT EXTRACTION OF PHYTOCHEMICALS FROM RIPE, RIPENING AND UNRIPE CARRISAA 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 unripe fruit extracts. Thus, ripe fruit showed best results in phytochemical analysis having good antioxidant content and highest antioxidant activity.

Key words: Phytochemicals; Carissa 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 (Arif et al., 2012). This research covered phytochemical analysis and gave no promotion to its cultivation (Aslam et al., 2014). 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 H2O2 (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°C for further phytochemical investigations.

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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 double distilled water at room temperature (25°C) 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, tannins, 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. All obtained extracts were stored in vials for further phytochemical and antioxidant investigations.

The reaction mixture was incubated in dark condition at 30°C 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,

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

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 30°C 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 acetic acid 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 95°C for 15 minutes. Then mixture was cooled at room temperature (25°C); added 1mL of ice cold 0.75% TCA, 3 mL Nash reagent, incubated at room temperature at 30°C for 15 minutes and absorption 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
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*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 CHCl₃ 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. Behil et al., (2019) performed qualitative analysis of methanolic extracts of *E. thyrsiflora*, *C. stockii* and *G. macrantha* and found maximum phytochemicals in those extracts that was further in agreement of present findings.

**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 (Onyelo 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.

<table>
<thead>
<tr>
<th>Qualitative test</th>
<th>Un-ripe fruit extracts</th>
<th>Ripening fruit extracts</th>
<th>Ripe fruit extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aq.</td>
<td>MeOH</td>
<td>E.A</td>
</tr>
<tr>
<td>Steroids (Red color)</td>
<td>+++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td><strong>Carbohydrates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molisch test (violet ring)</td>
<td>++++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Fehling test (Brick red)</td>
<td>-</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline reagent (yellow ppt)</td>
<td>-</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>NH₄OH (yellow ppt)</td>
<td>++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>Zn salt test (red color)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saponins (foam formation)</td>
<td>++++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Emomins: treated with NH₄OH and Benzene (red color)</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>Coumarins: treated with 10% NaOH (yellow color)</td>
<td>++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>Diterpenes: Copper acetate test (emrald green)</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Phytosterol: Salkowski test (golden red)</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Cardial glycosides: Keller-Killani Test (brown color)</td>
<td>++++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Phenol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaloids</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Xanthoproteic test (yellow ppt)</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Biuret test (violet pink)</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Amino acids (violet color)</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Anthocyanin (extracted treated with 2N HCl resulted violet after pink)</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
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.
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 H₂O₂ 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 carandas fruit by using free radical 1,1- diphenyl-2- picrylhydrazyl (DPPH) and found IC50 value 27.45±0.43 μg/mL. Supersarn 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; IC₅₀ 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 IC₅₀ 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 IC₅₀ 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.

References


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