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Amantagula Fruit (*Carissa macrocarpa* (Eckl.) A.DC.): Nutritional and Phytochemical Characterization

Fedia Souilem [1,2] · Maria Inês Dias [1] · Lillian Barros [1] · Ricardo C. Calhelha [1] · Maria José Alves [1] · Fethia Harzallah-Skhiri [2] · Isabel C. F. R. Ferreira [1]

Abstract

Fruits are one of the most promising food matrices and they have been explored in the discovery of new natural and safer bioactive compounds. *Carissa macrocarpa* (Eckl.) A. DC. fruits are widely consumed in African countries for the preparation of traditional foodstuff, but also for their beneficially health effects. Thus, as the authors' best knowledge there are no studies on the chemical and bioactive characterization of these fruits. Therefore, fruits of *C. macrocarpa* from Tunisia were chemically characterized regarding their nutritional value and bioactive compounds. Furthermore, the hydroethanolic extract of these fruits was evaluated regarding its bioactive properties. The fruit powder sample showed high amounts of sugars and polyunsaturated fatty acids (PUFA). The organic acids and tocopherols' profiles revealed the presence of five organic acids and two tocopherol isoforms, being quinic acid and α-tocopherol the most abundant. The hydroethanolic extract of the fruits presented high antioxidant, cytotoxic, anti-inflammatory, and antibacterial properties, showing activity against all the bacterial strains studied, also inhibiting the cell growth of all the tested tumor cell lines, with the exception of HepG2, and did not reveal toxicity for the non-tumor cells PLP2. Therefore, the fruits of *C. macrocarpa* could be included in a daily basis diet as a source of high nutritional quality compounds with high bioactive potential.

Keywords Amantagula fruits · Nutritional value · Chemical composition · Antioxidant · Antimicrobial · Cytotoxic · Anti-inflammatory

Introduction

There is a growing search for new sources of natural compounds, being derived directly or indirectly from fruits, the basis of many diets and one of the most promising food matrices, since they are generally consumed for their nutritional value, nutraceutical potential and health properties [1–3]. The round, and crimson fruits of *Carissa macrocarpa* (Eckl.) A.DC. shrubs, commonly known as Amantagula, are widely consumed by the local people of KwaZulu-Natal a province of South Africa, from where the species is native. *Carissa macrocarpa* from the Apocynaceae family, grows worldwide as ornamental including Saudi Arabia and Tunisia [4, 5]. Different morphological parts of *C. macrocarpa* are used in south Africa folk medicine to treat coughs and venereal diseases, also their leaves are used against diarrhea in livestock and the fruits have some effects against the human immunodeficiency virus (HIV) and hepatitis [6, 7]. *C. macrocarpa* fruits are also traditionally used in the preparation of jam, sauces, desserts, yogurt, jellies and ice cream [8].

It has also been proven that *C. macrocarpa* fruits are rich in vitamin C, chemical elements such as calcium, magnesium, iron, copper, among others, being also rich in essential fatty acids [4]. *Carissa macrocarpa* fruits are highly recommended for their anti-cariogenic properties due to its richness in

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✉ Isabel C. F. R. Ferreira
iferreira@ipb.pt

[1] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

[2] Laboratoire de Recherche "Bioressources": Biologie Intégrative & Valorisation (BIOLIVAL) LR14ES06, Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, Avenue Tahar Haddad, BP 74,5000 Monastir, Tunisia

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oleanolic acid and B-amyrin that can be used as a natural alternative to aspirin. It also presents hepatoprotective, hypoglycemic, anti-lipidemic and anti-proliferative effects [7].

Nevertheless, to the authors' best knowledge, no complete studies on the nutritional and phytochemical characterization of *Carissa macrocarpa* (Eckl.) A. DC. (amantagula fruits) have been found in literature. Therefore, in this study *C. macrocarpa* fruits obtained from Tunisia were chemically characterized regarding its nutritional value and bioactive compounds. Furthermore, the hydroethanolic extract of the fruits was evaluated for its antioxidant, antibacterial, anti-inflammatory, and cytotoxic properties.

Materials and Methods

Plant Material

Fully developed mature fruits were harvested in summer (August–September, 2016) from shrubs aged of 10 years, cultivated as an ornamental in a private garden, in Monastir (Latitude: 35°46.6794′ Nord, Longitude: 10°49.5702′ Est, elevation: 20 m), Tunisia. Voucher specimens (N° Cm1–5) identified by the botanist Pr. Fethia Harzallah-Skhiri, were deposited in the herbarium of the laboratory of Botany, High Institute of Biotechnology of Monastir. The fresh material was dried at 40 °C and then reduced to a fine powder and mixed to obtain a homogenate sample. The powder was stored at room temperature and protected from light, until further analysis. The sample was used for all the assays, which were then carried out in triplicate. The results were expressed as mean values and standard deviation (SD).

Nutritional Value and Chemical Characterization of Amantagula Fruits

Nutritional Value The dried fruits powder was analysed for proteins, fat, carbohydrates and ash according to the AOAC (Association of Official Analytical Chemists) procedures [9]. The crude protein content (N × 6.25, macro-Kjeldahl method) was determined following the AOAC 991.02; crude fat (Soxhlet apparatus with petroleum ether as extraction solvent) following the AOAC 989.05; ash content (incineration at 550 ± 15 °C) was determined using AOAC 935.42. Total carbohydrates (including fiber) were calculated by difference, using the following equation: Total carbohydrates (g/100 g) = 100 - (g fat + g protein + g ash). Total energy was calculated according to the following equation: Energy (kcal/100 g) = 4 × (g proteins + g carbohydrates) + 9 × (g fat).

Fatty Acids The dried fruits powder was also analysed for fatty acids determination, using a Soxhlet extraction of the samples to obtain a lipid fraction proceeded by a trans-esterification process. Identification and quantification was obtained by gas chromatography coupled with a flame ionization detector (GC-FID; DANI model GC 1000 instrument, Contone, Switzerland) following a procedure previously reported [10] and the results were expressed in relative percentage of each fatty acid.

Soluble Sugars The soluble sugars content determination was also performed in the dried sample following a procedure previously described by Dias et al. [10]. For that purpose, a HPLC system coupled to a refraction index detector (Knauer, Smartline system 1000, Berlin, Germany) was used for identification and quantification. Melezitose was used as an internal standard for quantification purposes. The results were expressed in g per 100 g of dry weight.

Organic Acids For organic acid determination the procedure described by Dias et al. [10] was followed, and determined using an ultra-fast liquid chromatography coupled to photodiode array detector (UFLC-PDA; Shimadzu Coperation, Kyoto, Japan). The quantification was performed by comparison of the peak area recorded at 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. For quantitative analysis a calibration curve with known concentration for each available organic acid, was constructed based on the UV signal. The results were expressed in g per 100 g of dry weight.

Tocopherols The four vitamers of tocopherols were determined also in the dried sample, following a procedure previously described by Dias et al. [10]. A HPLC (Knauer, Smartline system 1000, Berlin, Germany) coupled to a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) was used for that purpose. An internal standard (tocol) was used, for the quantification, based on the fluorescence signal response of each standard. The results were expressed in g per 100 g of dry weight.

Evaluation of Bioactive Properties

Extracts Preparation For hydroethanolic extract preparation, 1 g of the fruits powder was extracted by stirring with 30 mL of ethanol/water (80:20 *v*/v) at room temperature and 150 rpm, for 1 h. The extract was filtered through Whatman filter paper no. 4. The residue was re-extracted once more under the same conditions and both extracts were combined. Afterwards, the extracts were evaporated under vacuum (rotary evaporator Büchi R-210, Flawil, Switzerland) and further lyophilized. The lyophilized extract was re-dissolved in ethanol/water

(80:20, *v*/v) at 20 mg/mL for antioxidant activity, and in water at 100 mg/mL for antibacterial and at 8 mg/mL for anti-inflammatory and cytotoxicity evaluation, respectively. Several dilutions were obtained from the stock solutions depending on the assay.

Antioxidant Activity The antioxidant activity was evaluated by DPPH radical-scavenging activity, reducing power inhibition of β-carotene bleaching, and inhibition of lipid peroxidation using TBARS assay. The sample concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (according to DPPH, β-carotene/linoleate and TBARS assays) or after lecture of absorbance at 690 nm (reducing power assay) against sample concentrations [11]. Trolox was used as standard.

Antibacterial Activity The following Gram-negative bacteria: *Escherichia coli, Escherichia coli* ESBL, *Klebsiella pneumonia, Klebsiella pneumonia* ESBL, *Morganella morganii, Pseudomonas aeruginosa*, and Gram-positive bacteria: *Enterococcus faecalis, Listeria monocytogenes*, MSSA: Methicillin-sensitive *Staphylococcus aureus* and MRSA: methicillin-resistant *S. aureus*, were used. Minimum inhibitory concentration (MIC) were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay, following the methodology described by [2]. MIC was defined as the lowest extract concentration that prevented this change and exhibited inhibition of bacterial growth. Three negative controls were prepared, one with Mueller-Hinton Broth (MHB), another one with the extract, and a third with medium and antibiotic. One positive control was prepared with MHB and for each inoculum. For the Gram-negative bacteria, antibiotics, such as ampicillin and imipenem were used as positive controls, while ampicillin and vancomycin were used for the Gram-positive bacteria (Table A1, supplementary material).

Cytotoxic Activity MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) were used as human tumor cell lines and the cell density determination was performed using a sulforhodamine B assay previously described [11]. Results were expressed as the extract concentration that inhibited 50% of the net cell growth (GI_{50}), being calculated from the graph of sample concentrations against percentages of growth inhibition and expressed in μg/mL extract. For the non-tumor cells, a cell culture was prepared from a freshly harvested porcine liver (PLP2), following a procedure established by the authors [12]. Ellipticine was used as positive control and the results were expressed as GI_{50} values (sample concentration that inhibited 50% of the net cell growth) in μg/mL.

Anti-inflammatory Activity A mouse macrophage-like cell line RAW 264.7 stimulated with LPS was used in the assay and nitric oxide (NO) production was studied with Griess Reagent System kit as previously described [11]. Results were expressed as GI_{50} values (μg/mL) equal to the sample concentration providing a 50% inhibition of NO production. Dexamethasone (50 μM) was used as a positive control.

Results and Discussion

Nutritional and Chemical Composition of *C. macrocarpa* Fruits

The results regarding the proximate composition, soluble sugars and organic acids composition content of *C. macrocarpa* fruits are presented in Table 1. Carbohydrates and fat were the major macronutrients found. However, protein and ash were the lowest macronutrients.

Table 1 Proximate composition, soluble sugars and organic acids composition in *C. macrocarpa* fruits (mean ± SD, results expressed on fresh weight basis)

Moisture	78.83%
Nutritional value	g/100 g fw
Fat	3.53 ± 0.01
Proteins	0.74 ± 0.03
Ash	0.50 ± 0.09
Total available carbohydrates	16.40 ± 0.08
Energy contribution (kcal/100 g fw)	100.3 ± 0.2
Soluble sugars	g/100 g fw
Fructose	9.4 ± 0.3
Glucose	4.2 ± 0.2
Sum	13.5 ± 0.6
Organic acids	g/100 g fw
Oxalic acid	0.020 ± 0.002
Quinic acid	1.60 ± 0.02
Shikimic acid*	0.0021 ± 0.0001
Ascorbic acid*	0.0100 ± 0.0001
Citric acid	1.54 ± 0.03
Sum	3.17 ± 0.01

fw fresh weight basis. Calibration curves for organic acids: oxalic acid ($y = 9 \times 10^6 x + 45{,}973$, $R^2 = 0.9901$); quinic acid ($y = 610607x + 46{,}061$, $R^2 = 0.9995$); shiquimic acid ($y = 7\text{x}10^7 x + 175{,}156$, $R^2 = 0.9999$); ascorbic acid ($y = 7 \times 10^7 x + 60{,}489$, $R^2 = 0.9993$); citric acid ($y = 1 \times 10^6 x + 45{,}682$, $R^2 = 0.9997$). *Results expressed in mg/100 g

Wehmeyer et al. [13] reported a similar value of carbohydrate (16.4 g/100 g fw), protein (0.5 g/100 g fw), and ash (0.7 g/100 g fw) contents and lower values of fat contents (1.1 g/100 g fw) for amantagula fruits. Regarding the free sugars composition, fructose was the main soluble sugar found in the fruits followed by glucose. Our results were higher than those reported by Wilson and Dowen [14], where *C. macrocarpa* fruits from KwaZulu-Natal (South Africa) showed 56.12 mg/g dw of glucose and 59.26 mg/g dw of fructose, comparing with the present results, 198.3 mg/g dw and 444.02 mg/g dw of glucose and fructose respectively, calculated considering typical moisture contents of the fruits studied here (≈ 78.83%).

These differences could be explained by numerous factors, such as the different edaphoclimatic conditions between South Africa and Tunisia, the fruit genotype could be different, as also the nutritional status of the plant and postharvest treatments [13]. Also, sugar content and quantity can be changed according to the fruit maturity stage [15]. Five different organic acids (oxalic, quinic, shikimic, ascorbic and citric acid) were identified and quantified. Quinic and citric acids were the most abundant organic acids found in the fruits. All of these organic acids are of the upmost importance for the human metabolism since they are quickly absorbed into the blood circulation and beneficial for a healthy diet [16, 17]. Wehmeyer [13] described the organic acid profile in *C. macrocarpa* fruits from South Africa, except for vitamin C (ascorbic acid). However, the methodology applied to determined vitamin C content was different and for that manner it is not possible to compare the results obtained.

Results regarding fatty acids and tocopherols composition of *C. macrocarpa* fruits are given in Table 2. The fatty acids profile showed 18 different compounds, with monounsaturated fatty acids (61.6 ± 0.5%) being the major group present, mainly due to the presence of oleic acid (C18:1n9), followed by saturated fatty acids (23.0% ± 0.4%) due to the high content of palmitic acid (C16:0), and polyunsaturated fatty acid (15.46% ±0.05%) with a predominance of linoleic acid (C18:2n6). In general, the values obtained for fatty acids were slightly different to those mentioned in literature [4], for monounsaturated fatty acids (63.4%), being palmitoleic acid (C16:1, n-7) the main compound, followed by saturated fatty acids (30.7%) with myristic acid (C14:0) as the major compound, and polyunsaturated fatty acid with linoleic acid (C18:2, n-6) as the main compound [4]. Moreover, MUFA and PUFA had a beneficial effect on plasma lipid markers of cardiovascular disease risk and hypocholesterolemic potential comparing to SFA [18, 19]. In particular, Oleic acid has the capacity to decrease low-density lipoprotein (LDL) levels in blood, decrease blood pressure, suppress tumergenesis and ameliorate inflammatory diseases [20, 21].

These differences can be explained, as it occurs in other species and as already described above for sugars, by the different edaphoclimatic conditions of the regions (Tunisia and South Africa), but also by the different cultivars and ripening stages of the fruits [22]. Regarding tocopherols content, only two isoforms were identified in the fruits, being α-tocopherol the main vitamer, followed by γ-tocopherol. To the best of our knowledge, this is the first report to describe the content of vitamin E in these fruits. This vitamin is of the upmost importance for human nutrition and health through the prevention of many diseases including hypertension and cardiovascular diseases [23]. Especially α-tocopherol, which presents the highest biological activity, being based on the fact

Table 2 Fatty acids and tocopherols composition *C. macrocarpa* fruits (mean ± SD)

Fatty acids (relative percentage, %)			
Caprylic acid (C8:0)	0.045 ± 0.001	Eicosanoic acid (C20:1)	1.50 ± 0.05
Capric acid (C10:0)	0.079 ± 0.001	*cis*-11, 14, 17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0)	0.129 ± 0.002
Lauric acid (C12:0)	0.128 ± 0.003	Behenic acid (C22:0)	2.29 ± 0.01
Myristic acid (C14:0)	0.389 ± 0.001	Tricosanoic acid (C23:0)	0.155 ± 0.006
Penta-decanoic acid (C15:0	0).132 ± 0.008	Lignoceric acid (C24:0)	0.242 ± 0.007
Palmitic acid (C16:0)	13.2 ± 0.3	SFA	23.0 ± 0.4
Palmitoleic acid (C16:1)	0.259 ± 0.006	MUFA	61.6 ± 0.5
Heptadecanoic acid (C17:0)	0.306 ± 0.009	PUFA	15.46 ± 0.05
Oleic acid (C18:1n9)	59.8 ± 0.4	Tocopherols (mg/100 g fw)	
Linoleic acid (C18:2n6)	12.57 ± 0.04	α-Tocopherol	0.91 ± 0.01
α-Linolenic acid (C18:3n3)	2.77 ± 0.01	γ-Tocopherol	0.56 ± 0.03
Arachidic acid(C20:0)	1.03 ± 0.04	Sum	1.47 ± 0.04

fw fresh weight basis. Values in mean values ± standard deviation, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids

Table 3 Bioactive properties of hydroethanolic extract from *C. macrocarpa* fruits (mean ± SD)

Antioxidant activity	EC_{50} values (mg/mL)
DPPH scavenging activity	9.9 ± 0.6
Reducing power	1.59 ± 0.02
β-carotene bleaching inhibition	0.88 ± 0.08
TBARS inhibition	1.23 ± 0.04
Antibacterial activity	MIC values (mg/mL)
Gram-negative bacteria	
Escherichia coli	20
Escherichia coli ESBL	20
Klebsiella pneumoniae	>20
Klebsiella pneumoniae ESBL	>20
Morganellamorganii	20
Pseudomonas aeruginosa	20
Gram-positive bacteria	
Enterococcus faecalis	10
Listeria monocytogenes	20
MRSA	20
MSSA	20
Cytotoxic activity	GI_{50} values (μg/mL)
HeLa	66 ± 4
NCI-H460	57 ± 2
HepG2	>400
MCF-7	109 ± 5
PLP2 (non-tumor cells)	>400
Anti-inflammatory activity	GI_{50} values (μg/mL)
NO production inhibition	238 ± 4

EC_{50} values correspond to the extract concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values = 41 ± 1 μg/mL (DDPH), 18 ± 1 μg/mL (reducing power), 41.7 ± 0.3 μg/mL (β-carotene bleaching inhibition) and 23 ± 1 μg/mL (TBARS inhibition). MIC values correspond to the minimal extract concentration that inhibited the bacterial growth. *MRSA* Methicillin-resistant *Staphylococcus aureus*. *MSSA* Methicillin-susceptible *Staphylococcus aureus*. GI_{50} values correspond to the extract concentration achieving 50% of cell growth inhibition. Ellipticine GI_{50} value = 1.91 ± 0.06 μg/mL (HeLa), 1.03 ± 0.09 μg/mL (NCI-H460), 1.1 ± 0.2 μg/mL (HepG2), 0.91 ± 0.04 μg/mL (MCF-7), and 3.2 ± 0.7 μg/mL (PLP2). Dexamethasone GI_{50} value = 16 ± 1 μg/mL. *ESBL* extended spectrum β-lactamases

that they directly repair the oxidizing radicals, preventing the propagation of lipid peroxidation, and thus prevent the propagation of several diseases [24].

Bioactivity of *C. macrocarpa* Fruits

Results regarding the bioassays of the hydroethanolic extract obtained from *C. macrocarpa* fruits are presented in Table 3. Four antioxidant methods were chosen to determine several mechanisms of action. The extract showed antioxidant activity in all the assays, being more effective in the reducing power and TBARS inhibition assay, giving promising comparing to the positive control (Trolox), EC_{50} values of 0.88 ± 0.08 and 1.23 ± 0.04 mg/mL, respectively. There is no data regarding the antioxidant activity of *C. macrocarpa* fruits extract and others limited data on the antioxidant activity of *Carissa* species. It has been reported that the methanol and aqueous extract of *C. opaca* fruits presented EC_{50} values for the scavenging DPPH radicals of 0.077 mg/mL and 0.059 mg/mL, respectively. Also, Sahreen et al. [25] reported that the EC_{50} values for β-carotene bleaching assay of the extracts presented values of 433.45 μg/mL and 733.90 μg/mL, respectively, which are quite lower (higher antioxidant activity) than the values reported in the present work.

The antimicrobial effect of the extract was also determined using ten different bacterial species: Gram-positive (*Enterococcus feacalis, Listeria monocytogenes,* MRSA and MSSA) and Gram-negative (*Escherichia coli, Escherichia coli* ESBL*, Klebsiella pneumonia, Klebsiella pneumoniae* ESBL*, Morganella morganii and Pseudomonas aeruginosa)* ones. Considering the obtained data, it seems that the fruit was able to inhibit the growth of all the tested strains, including those with high antibiotic susceptibility (*E. coli* ESBL), except *K. pneumonia* and *K. pneumoniae* ESBL, with MIC values of 20 and 10 mg/mL. There are no reports in literature that evaluated the antibacterial potential of this fruit. Nonetheless, Moodley et al. [7] studied the antimicrobial activity of isolated compounds from *C. macrocarpa* fruits, revealing lower concentration than those reported herein. β-Amyrin, methyl oleanolate, and oleanolic acid presented MIC values ranging from 0.12 to 1.0 mg/mL and 3β-hydroxyolean-11-en-28,13β-olide presented MIC values of 0.06 to 0.12 mg/mL, for *K. pneumonia, E. coli* and *P. aeruginosa* [7]. However, it should be mentioned that the results presented herein were obtained with raw extracts, and not with the purified molecules. Moreover, the microorganisms used in this study are also clinical isolates, which present a higher resistance profile, in comparison with ATCC.

The fruit extract was able to inhibit the different human tumor cell lines studied, with the exception of hepatocellular (HepG2) carcinoma, being NCI-H460 (GI_{50} = 57 ± 2 μg/mL) the most susceptible to *C. macrocarpa* fruits, followed by HeLa (GI_{50} = 66 ± 4 μg/mL), and MCF-7 (GI_{50} = 109 ± 5 μg/mL) cell lines (Table 3). Moreover, no toxicity was observed for non-tumor porcine liver cells (PLP2), up to the maximal tested concentration (400 μg/mL) of the *C. macrocarpa* fruits. There are no reports on the cytotoxic activity of *C. macrocarpa* fruits. Those fruits also exhibited anti-inflammatory activity, but in comparison to dexamethasone, the extract revealed a low activity. Due to the lack of studies with *C. macrocarpa*, no comparisons could be

performed. However, the ethanolic extracts of *Carissa carandas* roots have been described as presenting anti-inflammatory activity against induced rat paw oedema [26].

This is novel study in the characterization of the nutritional properties and bioactive potential of the fruits of *C. macrocarpa*. The studied fruits showed higher amounts of sugars and polyunsaturated fatty acids than those reported by other authors, but the most importantly that was described here for the first time is the organic acids and tocopherols profile of *C. macrocarpa* fruits. Furthermore, the antioxidant, cytotoxic, anti-inflammatory and antibacterial properties of the hydroethanolic extract of *C. macrocarpa* fruits were studied for the first time. Considering all these bioactive effects, as also due to the fact that it did not reveal toxicity against non-tumor cells, it can be concluded that *C. macrocarpa* fruits could be included in a daily basis diet as a source of nutritional compounds with high bioactive capacity.

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Compliance with Ethical Standards

Conflict of Interest The authors state no conflict of interest.

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