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Bioactive Phytochemicals of *Carissa macrocarpa*: In Vitro and in Silico Investigations

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ABSTRACT

Carissa macrocarpa (Natal plum) is a tropical plant that is highly bioactive in nature. **Objectives:** To examine its phytochemical makeup and biological actions, and evaluate the impacts of the type of solvent and the level of fruit ripening. **Methods:** Ethanol and methanol were used in the extraction of ripening and unripe fruits. Extracts were evaluated for phytochemical constituents, antioxidant capacity (DPPH assay), antibacterial potential (disc diffusion and MIC), and phenolic profiling (HPLC). The identified compounds were further analyzed using molecular docking to predict anti-diabetic (DPP-4) and anti-cancer (EGFR) interactions. **Results:** Methanolic extracts, particularly from unripe fruits, showed higher yields and stronger antioxidant and antibacterial activities. Phytochemical screening confirmed the presence of flavonoids, terpenoids, saponins, and glycosides. HPLC revealed gallic acid and myricetin as dominant compounds. Docking studies suggest that these compounds have moderate binding affinities with DPP-4 and EGFR, indicating potential anti-diabetic and anti-cancer properties. **Conclusions:** The results have selected *C. macrocarpa* as a potential source of antioxidant and antibacterial agents. Molecular docking gives initial information as to its pharmacological diversity, and additional in vitro and in vivo research is obligatory before clinical use.

INTRODUCTION

Biodiversity is crucial in promoting the well-being of humans, livelihood, healthcare, and sustainable development [1]. The WHO reports that approximately 80 percent of the global population uses traditional medicine, which is plant-based, to receive primary healthcare [2]. More than half of the currently used drugs in a clinical setting are based on natural products [3]. The record of medicinal herbs is the oldest known record, dating back to 5,000 years ago in one of the Sumerian clay tablets in Nagpur [4]. Plant extracts prove to be quite efficient because they can be used in contact with certain receptors in the human body [5]. Numerous plants and fruits are not yet explored in terms of medicinal potential and open up opportunities of new treatment discoveries [6]. *Carissa macrocarpa*, or the Natal plum, is a popular traditional medicinal plant that belongs to the *Apocynaceae* family [7]. The genus of *Carissa* is native to tropical and sub-tropical areas of Africa, Australia, and Asia, where they can be found in South Africa and China [8]. It has different names, including *amatutungula* and *noemmoem*, which are used both as medicine and food [9]. Its bioactive compounds are rich in polyphenols, flavonoids, and vitamin C, and these include ellagic acid, kaempferol, and quercetin [10-12]. Although it has potential in treatment, little work has been done on the influence of ripening stages and solvents on the bioactive profile of *C. macrocarpa*. The hypothesis was

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that *Carissa macrocarpa* should show greater phytochemical diversity and better biological activities in the methanolic extracts, especially the unripe fruits, because of the polarity of the solvent and the composition of the metabolites of the immature tissues.

This study aims to identify pattern of phytochemical pattern, antioxidant as well and antibacterial potential of ethanol and methanol extracts of ripened and unripe *C. macrocarpa* fruits, along with molecular docking potential.

METHODS

This was an experimental laboratory-based study supported by in silico analysis conducted at the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore. The study duration was from October 2024 to June 2025. The ripened and unripe fruits were washed, cut into small pieces, and pre-dried under sunlight for 1 day (average ambient temperature 32 ± 2°C, relative humidity 55–60%). Subsequently, the samples were air-dried at room temperature (25 ± 2°C) for 5–6 days in a dust-free environment with adequate ventilation. Each drying batch was processed in triplicate to maintain reproducibility. The dried samples were then ground into fine powder using a sterile electric grinder and stored in airtight containers at 4°C until extraction. No human or animal subjects were involved in this study; therefore, ethical approval was not required. All experimental procedures complied with institutional, national, and international ethical guidelines for research involving plants. For extraction, 10 g of dried powdered plant material (ripened and unripe fruits) was mixed separately with 100 mL of solvent (70% ethanol or 100% methanol) in sterile flasks, maintaining a 1:10 (w/v) ratio. The mixtures were placed on an orbital shaker at 160 rpm for 24 h at room temperature and then filtered through Whatman No. 1 filter paper. The residues were re-extracted under identical conditions using 50 mL of the same solvent to ensure complete extraction of phytochemicals. Filtrates from both steps were combined, filtered through muslin cloth, and concentrated in a water bath at 75°C until dryness. The dried crude extracts were stored at 4°C until further use. Qualitative Phytochemical Screening: Standard procedures were followed for the phytochemical screening of all extracts to detect the presence of secondary metabolites. Tannins: 1mL of ferric chloride solution + 1mL of extract. Greenish- black color formation would indicate the positive results. Flavonoids: 1 mL of extract + a few drops of 10% NaOH solution. An intense yellow color appearance, which will turn colorless on the addition of dilute HCL, would indicate the positive results. Terpenoids: 2.5 mL of extract + 1 mL of chloroform + 1.5 mL of concentrated sulfuric acid. Reddish-brown would indicate the positive results. Carbohydrates: 1 mL of extract + 2 drops of Molisch's reagent + 1 mL of concentrated sulfuric acid. Violet colored ring at the interface of the extract of acid would indicate positive results. Reducing sugars: 2mL of Benedict's reagent + 1mL of extract. The resulting solution was boiled for 5 min in a water bath. A green, yellow, and brick red precipitation would indicate the presence of reducing sugars. Saponins: 1 mL of extract + 5 mL of distilled water, followed by vigorous shaking in separate test tubes. Stable froth formation for more than 10 min would confirm the presence of saponins. Alkaloids: A few drops of Wagner's reagent +1 mL of extract. The formation of a reddish-brown precipitate would indicate the presence of alkaloids. Glycosides: A few drops of 10% NaOH solution +1 mL of extract. The yellow color would indicate the presence of glycosides. Sterols: 1 mL of extract + 20 mL of chloroform + 30 mL of concentrated sulfuric acid. The presence of a reddish-brown color would indicate a positive result. The antibacterial testing of extracts was assessed by the Disc Diffusion Method by using *Escherichia coli* and *Bacillus subtilis*, and the antibiotic Erythromycin as a control. Inoculum of bacterial cultures was given on N-agar plates and incubated for 24h. Discs dipped in extracts and Erythromycin were mounted on agar plates. The agar plates were incubated at 37°C for 24h, after which inhibition zones were measured. Each extract and control (Erythromycin) was tested in triplicate (n = 3). Mean inhibition zone diameters ± SD were calculated. Minimum Inhibitory Concentration (MIC) was determined using the broth dilution method against *Bacillus subtilis*. Extracts were dissolved in DMSO, serially diluted (100 mg/mL to 1.5625 mg/mL) in LB broth, and inoculated with bacterial suspension. After incubation at 37°C for 24h, the optical density of each suspension was measured at 600nm. All concentrations were tested in triplicate, and the OD600 readings were averaged. The minimum inhibitory concentration (MIC) was objectively defined as the lowest extract concentration that resulted in a ≥90% reduction in bacterial growth compared to the growth control. The percentage inhibition was calculated using the following formula in this test: 3mL of the prepared DPPH reagent was added to 100µL of the samples in test tubes. Control was made by dissolving 3mL DPPH reagent and 100µL methanol in a test tube. The test tubes were incubated in the dark for 30 minutes. The optical density of the samples and the control was checked at 517nm. Each sample was analyzed in triplicate, and the results were expressed as mean ± SD. Ascorbic acid (0.1 mg/mL) was used as the standard antioxidant control. Antioxidant Activity = °(Absorbance of Control – Absorbance of Sample)/Absorbance of Control × 100. The HPLC analysis of the combined ethanolic extract was carried out to detect major phytochemicals. Three standards of flavonols (Quercetin, Kaempferol, Myricetin) and three standards of phenolics (Gallic acid, Caffeic acid, Sinapic acid) were used for this analysis. The HPLC method was validated by injecting each standard compound in

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triplicate. The mean retention time (RT) and the percentage relative standard deviation (%RSD) were calculated to ensure precision. Compound identification in the samples was based on the concordance of their RTs with the mean RT of the standards, accepting a deviation of less than ±2%. Phenolic and flavonoid compounds in the sample were identified by their comparison with standards. The docking protocol was validated by redocking the co-crystallized ligands (Nf7 for DPP-4, PDB ID: 4A5S; and AQ4 for EGFR, PDB ID: 1M17) into their respective active sites. The RMSD (root mean square deviation) values between the docked and crystallographic ligand conformations were calculated using PyMOL. An RMSD value of ≤ 2.0 Å was considered indicative of a reliable docking protocol [13]. The obtained RMSD values were within acceptable limits, confirming the accuracy of the docking parameters. All experimental assays were performed in triplicate (n = 3). Data were expressed as mean ± standard deviation (SD). All experimental assays were performed in triplicate (n=3), and data were expressed as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 27.0. For the extraction yield data, a two-way Analysis of Variance (ANOVA) was employed to determine the individual and interactive effects of fruit ripening stage (ripened vs. unripened) and solvent type (ethanol vs. methanol). For other assays comparing more than two groups, a one-way ANOVA was used, followed by Tukey's post hoc test for multiple comparisons. In molecular docking, each ligand-protein complex was docked in ten independent runs. The resulting binding energies (kcal/mol) were expressed as mean ± SD. A pairwise t-test was conducted to compare the mean binding energy of the phytochemicals against the mean binding energy of the respective control ligand (Nf7 for DPP-4, AQ4 for EGFR). Furthermore, based on established literature, binding affinities were classified using the following quantitative thresholds: ≤ -8.0 kcal/mol for "strong" binding, -8.0 < ΔG ≤ -6.0 kcal/mol for "moderate" binding, and > -6.0 kcal/mol for "weak" binding. In all analyses, a p-value of < 0.05 was considered statistically significant. Percentage Yield of Extracts: Weight of Dried Extract/Weight of Plant Material Used(g)/100

RESULTS

Out of all ethanolic and methanolic extracts, the methanolic extract of unripe Natal plums showed the maximum yield (Figure 1).

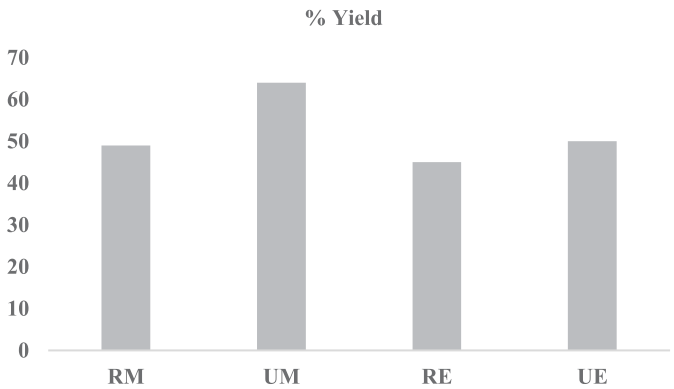

Figure 1: Extract Yield of Ethanolic and Methanolic Extracts of Ripened and Unripened Carissa macrocarpa

Phytochemical examination of the ethanolic and methanolic extracts of ripened and unripe Natal plums showed that they contained several secondary metabolites. All four extracts were positive in flavonoids, terpenoids, sterols, and glycosides (Table 1).

Table 1: Qualitative Phytochemical Composition of Ethanolic and Methanolic Extracts of Ripened and Unripe *Carissa macrocarpa*

Phytochemicals	UM	UE	RM	RE
Tannins	+ (Green Color)	+ (Green color)	–	–
Flavonoids	+ (Yellow Color)	+ (Yellow color)	+ (Yellow color)	+ (Yellow color)
Carbohydrates	+ (Violet ppt)	–	+ (Violet ppt)	–
Reducing Sugars	–	–	–	–
Terpenoids	+ (Reddish-Brown)	+ (Reddish-brown)	+ (Reddish-brown)	+ (Reddish-brown)
Alkaloids	–	–	–	–
Saponins	+ (Honeycomb Froth)	+ (Honeycomb froth)	–	+ (Honeycomb froth)
Glycosides	+ (Yellow Color)	+ (Yellow color)	+ (Yellow color)	+ (Yellow to orange)
Sterols	+ (Reddish-Brown Ring)	+ (Reddish-brown ring)	+ (Reddish-brown ring)	+ (Reddish-brown ring)

In the case of *Escherichia coli*, the highest zone of inhibition was recorded by RM (15 mm). Against *Bacillus subtilis*, the inhibition by the RM natal plums was maximum (18 mm). The usual commercial antibiotic standard, erythromycin, had an inhibition zone of 20 mm and 25 mm against *B. subtilis* and *E. coli*, respectively. These results show that different plant extracts have different antibacterial activity, with RM natal plum extract exhibiting the maximum antibacterial activity against both bacteria, especially against *Bacillus subtilis* (Table 2).

Table 2: Antibacterial Activity of Ethanolic and Methanolic Extracts of Ripened and Unripened Carissa Macrocarpa Against *Bacillus subtilis* and *Escherichia coli*

Bacterial Species	Samples	Inhibition Zones (mm)
	UM	15

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Bacillus subtilis (Gram-positive)	UE	12
	RM	18
	RE	17
	Erythromycin (Control)	25
Escherichia coli (Gram-negative)	UM	13
	UE	11
	RM	14
	RE	12
	Erythromycin (Control)	20

The MIC of the extracts against *Bacillus subtilis* was determined using the broth dilution method with a pre-defined inhibitory threshold of ≥90% growth reduction. The growth control (bacteria without extract) had a mean OD600 of 1.926. The unripe ethanolic (UE) extract demonstrated the greatest potency, with an MIC of 1.5625 mg/mL, at which concentration the mean OD600 was 0.073, corresponding to a 96.2% inhibition of bacterial growth (Figure 2).

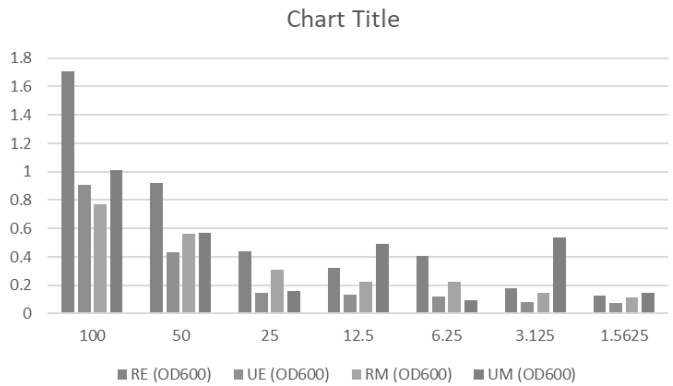

Figure 2: Minimum Inhibitory Concentration (MIC) of Extracts Against *Bacillus subtilis*

The UE extract demonstrated the highest antioxidant activity (79.74%) based on absorbance at 517 nm (Figure 3).

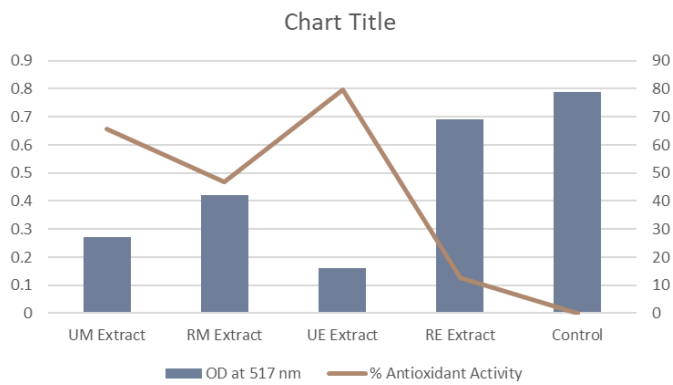

Figure 3: Antioxidant Activity of Ethanolic and Methanolic Extracts of Ripened and Unripened *Carissa macrocarpa*

UM: Unripe fruit methanolic extract, UE: Unripe fruit ethanolic extract, RM: Ripened fruit methanolic extract, RE: Ripened fruit ethanolic extract

Phenolic and flavonoid compounds in ethanolic and methanolic extracts of ripened and unripe Natal plums were identified using HPLC. Both phenolic acids (gallic acid, caffeic acid, and sinapic acid) and flavonoids (quercetin, myricetin, and kaempferol) were prepared as standard mixtures. Two peaks at 2.964 min and 11.623 min, denoting the gallic acid and sinapic acid, were observed in the RM extract, respectively. UM extract had one peak at 3.180 min, which was identified as gallic acid, and a gallic acid peak in the RE and UE extracts was at 2.962 min and 2.954 min, respectively. As far as flavonoids were concerned, the common peaks occurred at 4.016 min (quercetin), 4.988 min (myricetin), and 6.486 min (kaempferol). The extracts of UM had a peak at 5.000, RM had a peak at 5.015, UE had a peak at 5.002, all matched with myricetin, while RE had no peaks. The indication of the results is that myricetin is present in all samples except RE (Table 3).

Table 3: Retention Times of Phenolic Acids and Flavonoids in Ethanolic and Methanolic Extracts of Ripened and Unripened *Carissa macrocarpa* Determined by HPLC

Compound Type	Standard Compound	Mean RT (Standard) ± SD (min)	%RSD	RT in UM (min)	RT in UE (min)	RT in RM (min)	RT in RE (min)
Phenolic	Gallic Acid	3.114 ± 0.024	0.77	3.180	2.954	2.964	2.962
Phenolic	Caffeic Acid	5.683 ± 0.045	0.79	–	–	–	–
Phenolic	Sinapic Acid	10.795 ± 0.091	0.84	–	–	11.623	–
Flavonoid	Quercetin	4.016 ± 0.035	0.87	–	–	–	–
Flavonoid	Myricetin	4.988 ± 0.042	0.84	5.000	5.002	5.015	–
Flavonoid	Kaempferol	6.486 ± 0.058	0.89	–	–	–	–

The in silico molecular docking approach was conducted to ascertain the anti-diabetic and anti-cancer potentials of selected phytochemicals by using HPLC-profiling. For docking two compounds with two protein targets, DPP-4 (PDB ID: 4A5S) and EGFR (PDB ID: 1M17), the parameters, namely gallic acid and myricetin, were used with AutoDock Vina. The co-crystallized ligands (Nf7 with DPP-4 and AQ4 with EGFR) were used as controls to compare. A strong binding affinity was noted with myricetin, having a binding free energy equal to -8.9 kcal/mol, as against that of the control ligand (-7.4 kcal/mol) and a % effectiveness of 20.27%. In DPP-4, myricetin bound to it with a binding energy of -8.3 kcal/mol, and its effectiveness was 25.23 percent. Gallic acid had moderate interaction with both of the targets, with docking scores of -5.9 kcal/mol and % efficacy values of 46.85 and 20.27, respectively (DPP-4 and EGFR). These findings indicate that myricetin possesses a higher inhibitory capacity, especially on EGFR, and that gallic acid has a moderate effect on the anti-diabetic and anti-cancer activity of the plant extracts. The molecular docking results are summarized in Table 4. According to our pre-defined thresholds, myricetin demonstrated strong binding affinity against both EGFR (-8.9 kcal/mol) and DPP-4 (-8.3 kcal/mol). In contrast, gallic acid exhibited moderate binding affinity against both targets, with a docking score of -5.9 kcal/mol (Table 4).

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Table 4: Binding Affinities of Gallic Acid and Myricetin with DPP-4 and EGFR Targets Obtained from Molecular Docking

Sr. No.	Phytochemicals	Protein	Binding Energy (kcal/mol)	Control Ligand	Control Energy (kcal/mol)	% Effectiveness
1	Myricetin	DPP-4	-8.3	Nf7	-11.1	25.23%
2	Gallic Acid	DPP-4	-5.9	Nf7	-11.1	46.85%
3	Myricetin	EGFR	-8.9	AQ4	-7.4	20.27%
4	Gallic Acid	EGFR	-5.9	Aq4	-7.4	20.27%

DISCUSSION

The ethanolic yield was always lower than the yield of methanol extracts, which is a common observation in the phytochemical study of Carissa species and other plants belonging to the *Apocynaceae* family [10, 14]. The reason behind this difference is not only the increase in polarity of the solvent but also due to the fact that methanol has a higher capability of penetrating the tissues of plants, and has a greater capacity to dissolve polar as well as moderately non-polar constituents, which increases the efficiency of extraction. The difference in yield between unripe and ripe fruits could be because unripe fruits were more hydrated and contained more pectin, which could be interacting with solvents and creating the observed change in the yield, which is similar to those that were made by those who also identified the same effect in *Carissa carandas*. Screening of phytochemicals established that it contained plenty of flavonoids, terpenoids, saponins, glycosides, and sterols, which possess antioxidant and antimicrobial properties [14]. Tannin and carbohydrate specificity of the unripe fruits and methanolic extracts, respectively, may be indicative of different metabolite biosynthesis during fruit maturation, which is consistent with similar taxa [15, 16]. The ripened methanolic (RM) extract showed the best inhibitory effect on *Bacillus subtilis* and *Escherichia coli*, which is associated with its increased flavonoid content and the established disruption of the membrane by phenolics. On the other hand, the unripe ethanolic (UE) preparation exhibited lesser diffusion and greater MIC potency, suggesting the existence of greater, less mobile bioactive compounds, with more potent bacteriostatic activity. Dual behavior of such strong MIC and moderate zone diffusion has been reported in the phenolic-enriched fractions of *Carissa spinarum* and *C. carandas*. The antioxidant quality of the UE extract can be attributed to the fact that it preserves heat-sensitive polyphenols and anthocyanins, which are lost during ripening. This is in line with previous research studies that have indicated a negative correlation between ripening and the total phenolic concentration in the fruits of Carissa. HPLC analysis established the presence of myricetin, gallic acid, and sinapic acid, which are all bioactive compounds with reported bioactivities. Myricetin has a good binding affinity with EGFR (-8.9 kcal/mol), which is confirmed by our molecular docking results. This is a better value than the -7.8 kcal/mol that myricetin was found to have against the same target in a previous docking study [17], which indicates a possibly greater inhibitory activity. Likewise, its affinity towards DPP-4 (-8.3 kcal/mol) is close to the -8.5 kcal/mol reported in a separate in silico study of antidiabetic compounds against myricetin [18-20]. These specific numerical comparisons strengthen the computational evidence for myricetin as a multi-target lead compound."

CONCLUSION

The study at hand revealed that the ripened and unripe *Carissa macrocarpa* (Natal plum) ethanol- and methanol-extracts have a rich source of bioactive secondary metabolites, such as phenolics and flavonoids, and that they may be used in antioxidant and antibacterial applications. HPLC profiling established the presence of compounds, including gallic acid and myricetin, and molecular docking analyses indicated their possible interaction with important biological targets (EGFR and DPP-4) with possible implications in anticancer and antidiabetic activities.

Authors Contribution

Conceptualization: TI
Methodology: TI, JA, LA
Formal analysis: TI
Writing review and editing: TI, JA, LA, DQ, TM

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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