

SCREENING OF ANTICHOLINESTERASE AND ANTIOXIDANT ACTIVITIES OF *CARISSA CARANDAS L.* FRUIT EXTRACT FOR ALZHEIMER'S TREATMENTKARTHIGADEVI KAMALAKANNAN^{1,2}, SHANMUGANATHAN SEETHARAMAN^{3*},
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ABSTRACT

Objectives: The aim of this study was to evaluate the acetylcholinesterase (AChE) c inhibitory and antioxidant activity of unripened fruits of *Carissa carandas* extract to introduce new source for the management of Alzheimer's disease.

Methods: Preliminary phytochemical screening identified the existence of flavonoids, phenolics, alkaloids, sterols, and saponins. The evaluation of antioxidant activity was led through 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP) capacity, and anti-cholinesterase activity by the Ellman method.

Results: According to the obtained results, the extract contained a valuable source of flavonoids and phenolic compounds and has also shown AChE inhibition activity, which reveals the good inhibition potential with half-maximal inhibitory concentration of $95.92 \pm 1.05 \mu\text{g/mL}$ and significant antioxidant activity in DPPH assay and FRAP assay compared to the respective standards.

Conclusion: The present study revealed that the *C. carandas* fruit extract showed potential AChE inhibition and antioxidant activity. However, further investigations on the identification of active components in the extracts are needed.

Keywords: Acetylcholinesterase, *Carissa carandas*, Total phenolic, Flavonoid compounds, Antioxidant.

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative ailment linked to the aging process. This irreversible brain disorder hampers daily functioning by causing memory loss and cognitive decline [1,2]. The Journal of Alzheimer's Association 2024 highlights the influence of AD on public health that includes 7.2 million individuals of 65 years and older who are thought to have AD at this time. If no medical advances are made to prevent or treat AD, this figure may increase to 13.8 million by 2060. According to official AD death certificates, 120,122 AD deaths occurred in 2022. AD has been the seventh largest cause of death in the United States since 2020, but according to data from 2023, AD is probably going to reclaim its position as the sixth most common cause of death. Fatalities from human immunodeficiency virus, heart disease, and stroke declined between 2000 and 2022, whereas recorded fatalities from AD climbed by about 142% [3]. As the exact cause of AD is still a mystery. Number of factors, including as diminished acetylcholine (ACh) levels, oxidative damage, buildup of misfolded amyloid- β , and biometal imbalance, play a key role in the progression of AD. The mechanism of AD has been explained by a number of ideas based on these aspects [4,5].

The "cholinergic hypothesis" recognizes that both acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) can hydrolyze the neurotransmitter ACh into choline and acetate, thereby terminating the signal at the synapse. It is acknowledged that inhibiting both AChE and BuChE is essential for effectively treating AD by increasing the ACh distribution in various regions of the brain [6,7].

Currently, galantamine, donepezil, and rivastigmine are examples of AChE inhibitors that can be used to treat people with mild to moderate AD. For those experiencing moderate to severe stages, N-methyl D-aspartate receptor antagonists (NMDA), such as memantine, are prescribed. These treatments aim to improve the quality of life for Alzheimer's patients and can temporarily slow the progression of dementia symptoms.

Nowadays, natural ingredients are the source of the majority of medications that delay the progression of illness. The anti-inflammatory, antibacterial, and antioxidant qualities of plant extracts and phytochemicals are widely recognized and can play a significant role in therapeutic interventions [8,9].

The Apocynaceae family includes the evergreen shrub *Carissa carandas*, which is still understudied as commercial, economic, medicinal, and nutritional potential. Other names for it include Ci-Huang-Guo (Mandarin Chinese), Karamcha (Bengali), Karonda (Devanagari), and "Crane berry" (English). It is discovered to be widely dispersed throughout Bangladesh, Nepal, Sri Lanka, India, Pakistan, and Myanmar's tropical and subtropical regions. Berries from *C. carandas* are renowned for their role in traditional medicine, where they have been employed to address a range of ailments such as muscle spasms, swelling, diarrhea, and epilepsy [10]. These fruits are also recognized for their potential benefits in treating hemorrhoids, loss of appetite, and nerve disorders (as a nervine), as well as conditions such as colic, enlarged spleen, enlarged liver, absence of menstruation, heart diseases, and mental anorexia. The

roots of *C. carandas* are utilized to alleviate itching, act as a vermifuge, and serve as a bitter tonic for the stomach. Recent pharmacological studies have substantiated the traditional claims regarding *C. carandas*, confirming its antibacterial [15], anti-inflammatory [12], anticancer [13], antidiabetic [14], and antioxidant [11] properties. Extracts from *C. carandas* fruits have been found to encompass anthocyanins, phenolic acids, flavonols, alkaloids, and terpenoids [16,17]. The plant is a useful source because of these chemicals, which also contribute to its high biological activity.

However, the biological potential of the plant was assessed in the previously described experiments using crude extracts.

This study seeks to pinpoint and examine the phytochemical constituents and investigate the potential AChE inhibitory and antioxidant characteristics of *C. carandas* fruit extracts. The objective is to underscore the importance of these plants as possible sources for creating therapeutic agents for AD.

METHODS

Solvents and reagents

Standard extraction solvents, such as gallic acid and quercetin, were used; they were all purchased from the Sigma Aldrich local supplier. Except as indicated, every chemical, reagent, and solvent used in this investigation was of analytical grade.

Plant collection and sample preparation

C. carandas fruits were collected in the month of September 2023 from Amrithi Hills, located within the Amrithi Zoological Park near Vellore, Tamil Nadu, at approximately 12.732363°N, 79.056673°E, with the main entrance near Amrithi village (approx. 25 km from Vellore). Plant species have been identified and authenticated by Dr. Sivasankar, Botanist and Taxonomist, Auroville Botanical Services, Auroville, Tamil Nadu, India. The herbarium specimen with voucher number 13524 was placed in the herbarium of Auroville; shown in figure 1. After the fruits were cleaned, the fruits were cut into slices. The plant materials were then air-dried at 25–30°C temperature for 14 days, weighed, powdered, and stockpiled in the dark at –20°C until further processing.

Plant extraction

$$\text{Yield Ratio} = \frac{\text{Plant Extract Weight}}{\text{Dried Plant Sample Used Weight}} \times 100$$

The powdered form of 250 g of dried unripe *C. carandas* fruits underwent a hot extraction process using a Soxhlet apparatus with Ethanol as the solvent. The extraction process was carried out for 24 h. To remove the solvent, a rotary evaporator and a water bath were used to evaporate the solvents, after that the resulting substance was kept at –20°C for later use, enabling the calculation of the percentage yield [18].

Phytochemical screening of the *C. carandas* fruit extract (qualitative analysis)

Using standard methods, major compounds including alkaloids by Mayer's test, flavonoids by Alkaline reagent test, phenols by Lead acetate test, steroids by Salkowski test, and saponins by froth test were discovered to be present in the ethanolic extract of *C. carandas* fruits (EECCF) as confirmed by qualitative phytochemical analysis [19,20].

Phytochemical screening of the *C. carandas* fruit extract (quantitative analysis)

Total phenolic contents estimation

The colorimetric technique known as Folin-Ciocalteu (FC) used gallic acid as the standard reference, with ethanol serving as the blank to evaluate the total phenolic content. The process involved combining the test sample at a concentration of 1 mg/mL and 0.2 mL of the FC reagent (diluted 1:1) with 0.5 mL of water. After 5-min interval, a saturated sodium carbonate solution (8% w/v in water) was added in 1 mL, and pure water was used to get the mixture's final volume down to 5 mL. At

765 nm, the blue color's absorption was examined. After allowing the reaction to develop in the dark for 30 min, with three separate readings were taken from different samples. The phenolic content was expressed as Gallic acid equivalents (GAE) per gram dried extract, according to the gallic acid standard curve (10–320 µg/mL); all measurements were performed in triplicate [21,22]. Using the following calculation, from the calibration curve, gallic acid (mg/g) was used to represent the total phenolic concentration

$$y = 0.0097x + 0.0046, R^2 = 0.9995$$

Estimation of total flavonoid contents

The method described by Ali *et al.* [22,23] was employed to evaluate the plant extracts' overall flavonoid content. A mixture was prepared by combining a 2% aluminum chloride in ethanol solution in 0.5 mL with the test sample in 1 mL, which had a concentration of 1 mg/mL. The mixture should be allowed to stand at room temperature for 1 h, and the absorbance level was determined at 420 nm. The emergence of a yellow tint suggested the presence of flavonoids. Quercetin, with concentrations ranging from 10 to 320 µg/mL, was used as a reference to create a calibration curve. Using the following formula from the calibration curve, quercetin (mg/g) was used to represent the total flavonoid concentration.

where, y represented the absorbance. Each measurement was conducted 3 times.

$$y = 0.0131x + 0.0526, R^2 = 0.9975$$

In vitro antioxidant activity

The inhibitory effect of *C. carandas* fruit extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) was evaluated using a method previously described [24,25]. A 1 millimole of DPPH stock solution was prepared in methanol and kept at –20°C for later use. To prepare a 0.1 mM DPPH solution, methanol (90 mL) was used to dilute 10 mL of the original solution. 2 mL of DPPH, 2 mL of methanol, and 0.2 mL of the extract were combined in test tubes and kept in the dark for 30 min. After incubation, the absorbance was measured with a spectrophotometer at 517 nm. Methanol served as the blank, while quercetin (125 µg/mL) was employed as the standard for measurements. The below formula was used to determine the proportion of inhibition of DPPH.

$$\text{The ratio of inhibition} = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \times 100$$

The ferric-reducing antioxidant power (FRAP) capacity of *C. carandas* fruit extracts was calculated following the described methodology by Zahin *et al.* [26,27]. The procedure involved mixing 300 µL of the FRAP working solution with 100 µL of the extract, prepared at a concentration of 1 g/20 mL. This mixture is composed of a 300 mmol/L acetate buffer at pH 3.6, 10 mmol/L of 2,4,6-tripyridyl-s-triazine dissolved in 40 mmol/L hydrochloric acid, and 20 mmol/L ferric chloride, combined together at 10:1:1 ratio. For half an hour, the solution was incubated at 20°C–25°C. A spectrophotometer was then used to detect absorbance at 596 nm, and the findings were reported as Fe²⁺ mmol/g of dried extract. All tests were performed in triplicate. Data were analyzed with the GraphPad Prism 6.0 software, using a variance model and Tukey's test at p < 0.05.

In vitro AChE enzyme inhibitory activity

The spectrophotometric technique described by Ellman *et al.* [28] was modified to evaluate AChE activity. The source of AChE enzyme by electric eel. To adjust for the nonenzymatic degradation of Ach, a blank cuvette was set up containing 275 µL of 0.1 M potassium phosphate at pH 8, 500 µL of a 3 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution (also in 0.1 M potassium phosphate at pH 8), 100 µL of 15 mM acetylthiocholine (dissolved in water), and 100 µL of the sample under test at concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 200 µg/mL, and 400 µg/mL. In the reaction cuvette, 25 µL of the buffer was substituted with 0.16 U/mL of AChE solution. The

resulting solutions 1 mL were then analyzed using a spectrophotometer. Ach undergoes hydrolysis to form thiocholine, which quickly reacts with DTNB to produce yellow substance. The reaction was seen for 5 min at a wavelength of 405 nm, and the absorbance was recorded. Galantamine, at concentrations of 25, 50, 100, 250, and 500 µg/mL, was considered the positive control, whereas the negative control was the reaction mixture that did not contain the plant sample. The percentage inhibition was calculated using the specified formula, and half-maximal inhibitory concentration (IC₅₀) values were determined. All tests were performed in triplicate. Data were analyzed with the GraphPad Prism 6.0 software, using a variance model and Tukey's test at p<0.05.

$$\text{Inhibition Ratio} = \frac{[(\text{OD of control} - \text{OD of test}) / (\text{OD of control})] \times 100}{100}$$

RESULTS

Extraction yield

Extraction of unripe *C. carandas* fruit resulted in a yield of 25.83% (w/w).

Phytochemical analysis and antioxidant activity

The EECCF underwent phytochemical analysis, as detailed in Table 1, revealing the existence of tannins, phenolics, flavonoids, alkaloids, phytosterols, and saponins in the fruit extract.

Data provided in Table 1 indicate that the extract showed the presence of Alkaloids by Mayer's test, Flavonoids by Alkaline reagent test, Phenols by Lead acetate test, Steroids by Salkowski test, and Saponins by froth test.

Results are expressed as mean±standard deviation (n=3). The means followed by different letters differ by the Tukey test at *p<0.05; the data provided in Table 2 represent the quantity of phenolic and flavonoidal compounds present in per gram of extract, which was compared with the respective standards. GAE refers to Gallic acid equivalent, and quercetin equivalent (QE) refers to QE.

According to the quantitative analysis, the extractive assays indicated that the total phenolic compounds amounted to (43.37±0.01 mg GAE/g dried extract) and the flavonoid content was (2.42±0.02 mg QE/g dried extract), as detailed in and Table 2. Consequently, the extract demonstrated a higher concentration of total phenolic compounds.

Data are presented as the mean±SD (n=3). Different letters (b–d) for each column symbolize significant differences (p<0.05) by means of Tukey's test. ^aQuercetin- Reference Standard. Table 3 shows that the extract from *C. carandas* fruit demonstrated a significant 73.2±2.01% of DPPH scavenging capacity which corresponds to 88.5±2.12% of quercetin. Likewise, in the FRAP assay, the *C. carandas* fruit extract achieved a FRAP value of 51.5±0.61 Fe²⁺mmol/g, equivalent to 59.2±0.06Fe²⁺ mmol/g of quercetin, emphasizing its promise as an organic source of antioxidants. The results were stated in terms of equivalents of quercetin, calculated by comparing the findings to a standard curve based on known quercetin concentrations.

EECCF was assessed using the popular Ellman technique for AChE inhibition at varying concentrations. The percentage of AChE inhibition by the extractives is shown in Fig. 2 and Table 4. The AChE enzyme was dose-dependently inhibited by all test extract doses. Galantamine was used as the reference standard.

Results are expressed as mean±standard deviation (n=3). The means followed by different letters differ by the Tukey test at P<0.05; ^aGalantamine –a Reference Standard; EECCF revealed a strong AChE inhibitory capacity, with clinically relevant IC₅₀ values (Table 5), although in comparison with the positive control, galantamine, had a higher IC₅₀ value. IC₅₀ values were calculated from dose-response curve using linear regression analysis.

Table 1: Qualitative phytochemical analysis of *Carissa carandas* fruit extract

S. No.	Test name	Observation	EECCF
1	Alkaloids by Mayer's test	reddish brown precipitate	Presence of Alkaloids
2	Flavonoids by Alkaline reagent test	formation of intense yellow color	Presence of Flavonoids
3	Phenols by Lead acetate test	A bulky white precipitate	Presence of Phenols
4	Steroids by Salkowski test	The upper layer turns pink color	Presence of Steroids
5	Saponins by froth test	Copious lather formation	Presence of Saponins

EECCF: Ethanolic extract of *Carissa carandas* fruits

Table 2: Phytochemical contents of *Carissa carandas* fruit extracts

Sample	Total phenolic compound (mg GAE/g of extract)	Total flavonoid content (mg QE/g of extract)
EECCF	43.37±0.01*	24.2±0.02*

GAE: Gallic acid equivalents, EECCF: Ethanolic extract of *Carissa carandas* fruits, QE: Quercetin equivalent. Results are expressed as mean ± standard deviation (n = 3). The means followed by different letters differ by the Tukey test at *P<0.05

Table 3: The antioxidant activity using DPPH and FRAP radical scavenging assays of *Carissa carandas* fruit extract

Fruit extracts/ standards	DPPH Scavenging capacity (%)	FRAP (Fe ²⁺ mmol/g)
EECCF	73.2±2.01 ^b	51.5±0.61 ^c
^a Quercetin	88.5±2.12 ^c	59.2±0.06 ^d

DPPH: 2,2-diphenyl-1-picrylhydrazyl, FRAP: Ferric-reducing antioxidant power, EECCF: Ethanolic extract of *Carissa carandas* fruits. Results are expressed as mean ± standard deviation (n = 3). The means followed by different letters differ by the Tukey test at *P<0.05

Table 4: Percentage inhibition of *Carissa carandas* fruit extract on AChE enzyme activity

Concentration (µg/mL)	EECCF (% inhibition)	Galantamine (% inhibition)
25	25.19379845	2.60747
50	33.12191684	15.539112
100	52.8893587	32.276251
200	72.90345314	54.263566
400	93.16420014	77.202255

EECCF: Ethanolic extract of *Carissa carandas* fruits, AChE: Acetylcholinesterase. Data are presented as the mean ± SD (n = 3). Different letters (b–d) for each column symbolise significant differences (p < 0.05) by means of Tukey's test. ^aReference Standard

Table 5: IC₅₀ value of AChE inhibition activity of *Carissa carandas* fruit extract

Sample	IC ₅₀ value of AChE inhibition
EECCF(µg/mL)	95.92±1.05 ^b
^a Galantamine (µg/mL)	41.72±0.08 ^c

IC₅₀: Half-maximal inhibitory concentration, EECCF: Ethanolic extract of *Carissa carandas* fruits, AChE: Acetylcholinesterase. Results are expressed as mean ± standard deviation (n = 3). The means followed by different letters differ by the Tukey test at P<0.05; ^aReference Standard

DISCUSSION

AD, a progressive neurological disorder, is the most common type of dementia found in elderly individuals. There is currently no proven

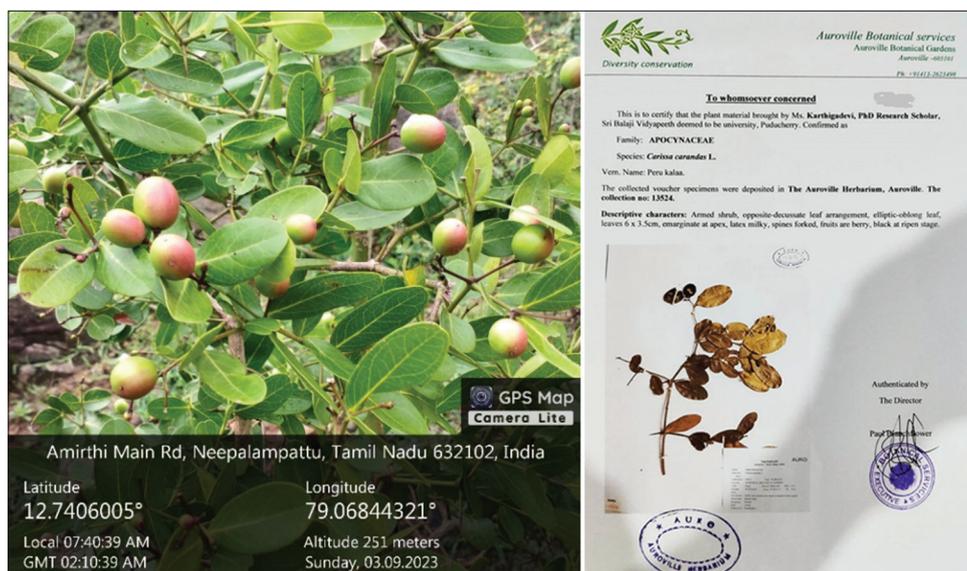


Fig. 1: *Carissa carandas* plant collection and authentication

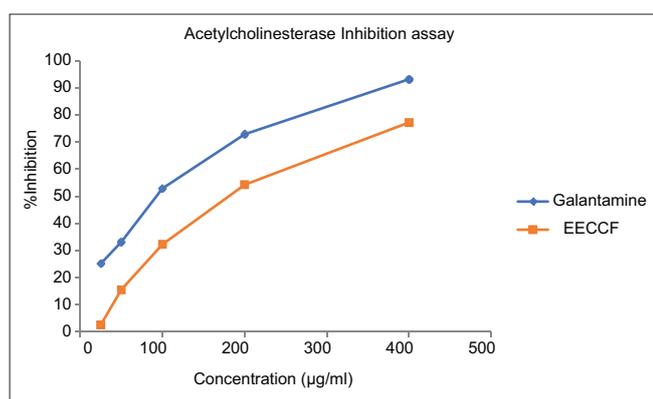


Fig. 2: Percentage inhibition of acetylcholinesterase enzyme activity demonstrated by the *Carissa carandas* fruit extract and galantamine

cure for AD. Around 50 million individuals worldwide suffer from AD, and by 2050, that number will have tripled. AD has grown to be a major health issue among the elderly as a result of population growth and longer lifespans. As of right now, the Food and Drug Administration has only approved three cholinesterase inhibitors for AD cure: Galantamine, donepezil, rivastigmine, and memantine, a partial NMDA receptor antagonist [29]. These medications only slightly delay the disease's course and provide symptomatic relief; they do not halt AD's progression [30].

We created new medications as a result of the limited number of existing ones and their usage restrictions. Plants have already shown themselves to be a significant source of several drug classes, and new AD candidate medications have been created. With several biological properties, *C. carandas* is a traditional medicinal herb used in our present study [31]. In this study, we report the *in vitro* cholinesterase inhibitory and antioxidant activities of *C. carandas* fruits.

Different kinds of phytochemicals found in plants support biological activity. In this research, Ethanol was used as the solvent for the extraction of the *C. carandas* fruits. Total phenolic (43.37 ± 0.01 mg GAE/g) and flavonoid (24.2 ± 0.02 mg QE/g) contents were found to be greater in *C. carandas* extract. Flavonoids and phenols are important classes of secondary metabolites with a lot of biological activity that are found in plants. They are referred to as natural antioxidants due

to their capacity to neutralize free radicals by providing electrons or hydrogen.

Our plant extracts were also used to assess the antioxidant capacity utilizing the DPPH and FRAP tests. The DPPH test is simple, accurate, and effective method of evaluating plant-based extracts' ability to scavenge radicals. The method involves measuring how the presence of antioxidant chemicals causes the DPPH coloration to change from violet to light yellow. Antioxidant activity in the current investigation was found to be in line with phytochemical potential. Table 2 confirms the findings of this study by showing a consistent ability to inhibit free radicals and achieving the highest level of inhibition when compared to standard quercetin.

The FRAP assay is a widely recognized method for determining the total antioxidant capacity of experimental extracts. This test evaluates an extract's ability by seeing how ferric ions change into ferrous ions, to contribute electrons. In current research, the FRAP values were found to range from $51.5 \pm 0.61 \text{Fe}^{2+}$ mmol/g, in contrast with the typical quercetin value of $59.2 \pm 0.06 \text{Fe}^{2+}$ mmol/g. This indicates that the extract exhibits significant antioxidant properties.

Cholinesterase inhibition remains the most promising treatment approach for AD medication development. AChE inhibitors enhance cholinergic neurotransmission, raise Ach levels at synapses, and enhance animal memory and cognition [32]. Medicinal herbs are used to treat AD because they include a variety of chemical components that have the ability to inhibit cholinesterase [33]. Because natural chemicals have fewer harmful effects, there is now more interest in natural AChE inhibitors.

In our study, we report for the 1st time the AChE inhibitory activity of *C. carandas*. Although numerous plants used in traditional medicine for memory enhancement have been studied, only a limited number have demonstrated significant AChE inhibitory potential [33]. The present AChE inhibition report was compared to the previous reported plants, such as *Baliospermum montanum*. *Baliospermum montanum* showed IC_{50} value as 137.5, *humboldtia brunonis* wall. var. *raktapushpa* showed IC_{50} value as 105.7, *Pittosporum viridulum* showed IC_{50} value as 128.3 [34] and *Withania somnifera* showed IC_{50} value as 124.0 [35].

Whereas the extract from *C. carandas* fruit showed superior AChE inhibition. During our evaluation, the IC_{50} value of EECCF was determined to be $95.92 \mu\text{g/mL}$. These findings indicate that the *C. carandas* fruit extract exhibits notable AChE inhibitory activity, likely

due to its flavonoid and phenolic content. Flavonoids, an omnipresent class of polyphenolic compounds, are commonly present in fruits, vegetables, and plant-derived beverages. Owing to the numerous inevitable biotic properties of flavonoids, they might act as anticancer, antioxidant, anti-inflammatory, antimicrobial, and antiviral agents. In addition, flavonoids have shown neuroprotective effects in many clinical trials [36].

During AD pathogenesis, multiple signaling pathways are involved and targeting a single pathway may relieve the symptoms but not provides the permanent cure. Flavonoids communicate with different signaling pathways and adjust their activities, accordingly prompting valuable neuroprotective impacts. The ability of flavonoid and phenolic compounds present in the extract bind directly to the AChE enzyme, thereby preventing the breakdown of the neurotransmitter acetylcholine (ACh) in the brain. This mechanism is a key therapeutic strategy for treating neurodegenerative conditions like AD by enhancing cholinergic signaling. Flavonoid-like phytochemicals likewise hamper the movement of obsessive indications of neurodegenerative disorders by hindering neuronal apoptosis incited by neurotoxic substances [37]. Hence, phytochemicals such as flavonoids, phenolic compounds, and their neuroprotective properties could be used as a potential source for the treatment of AD.

CONCLUSION

The current research revealed that extracts from *C. carandas* fruit significantly inhibit cholinesterase activity and possess antioxidant properties, which may be beneficial in managing AD. The findings suggest that further evaluation of the identification of active components in this plant and *in vivo* animal models is justified to explore the potential of the selected plant extract for the management of AD.

ETHICAL APPROVAL

Not applicable.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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