

Original Research Article

Phytochemical screening, antinociceptive, anthelmintic and cytotoxicity studies of the leaves of *Carissa carandas* Linn. (Family: Apocynaceae)

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Received: 14 March 2018

Accepted: 04 April 2018

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ABSTRACT

Background: *Carissa carandas* Linn. (Bengali name- Karamya, Karancha, Karamcha, English name-Karaunda) commonly known as Karanda is a widely used medicinal plants belongs to Apocynaceae family. The various plant parts of *C. carandas* have been used for ethnomedicine in the treatment of human diseases, such as diarrhea, stomachic, anorexia, intermittent fever, mouth ulcer and sore throat, syphilitic pain, burning sensation, scabies and epilepsy. The current studies were aimed to investigate the phytochemical constituents, antinociceptive, anthelmintic and cytotoxic activities of the methanol extract of leaves of *C. carandas*.

Methods: The antinociceptive property of the methanol leaves extract of *C. carandas* was evaluated in swiss albino mice by using acetic acid-induced writhing test and anthelmintic activity of the fresh leaves juice was performed by observing the time of paralysis and the time of death of earth worms *Pheretima posthuma*. On the other hand, the methanol extract of leaves of *C. carandas* was screened for cytotoxic activity by brine shrimp lethality bioassay.

Results: Phytochemical screening showed that the methanol leaves extract contained alkaloids, steroids, flavanoids, tannins, saponins and reducing sugar. The antinociceptive activity test revealed that the extract showed significant antinociceptive activity and the fresh juice of the leaves of *C. carandas* showed potent anthelmintic activity. In cytotoxicity studies, the methanol leaves extract displayed moderate cytotoxic activity when compared with standard drug, vincristine sulphate.

Conclusions: In the current studies, these observations also support the use of this plant for medicinal purposes and encourage further investigations for more fruitful results.

Keywords: *Carissa carandas*, Phytochemical screening, Antinociceptive, Anthelmintic, Cytotoxicity

INTRODUCTION

Carissa carandas Linn. has been used as a traditional medicinal plant over thousands of years in the Ayurvedic, Unani and Homoeopathic system of medicine. *C. carandas* commonly known as Karamya, Karamcha, Karancha (Beng.), Karaunda (Eng.) belonging to Apocynaceae family has been used traditionally to treat different ailments. It is a climbing much-branched shrub with sharp hard thorns and milky latex-rich oblong fruits, planted in almost all the districts in Bangladesh.¹ It usually grows to 10 or 15 ft (3-5 m) high, sometimes

ascending to the tops of tall trees; and rich in white, gummy latex. Those are branches, numerous and spreading, forming dense masses, are set with sharp thorns, simple or forked, up to 2 in. (5 cm) long, in pairs in the axils of the leaves. The leaves are evergreen, opposite, oval or elliptic, 1 to 3 in (2.5-7.5 cm) long; dark-green, leathery, glossy on the upper surface, lighter green and dull on the underside. The fragrant flowers are tubular with 5 hairy lobes, which are twisted to the left in the bud instead of to the right as in other species. They are white, often tinged with pink, and borne in terminal clusters of 2 to 12. The fruit, in clusters of 3 to 10, is

oblong, broad-ovoid or round, 1/2 to 1 in. (1.25-2.5 cm) long; has fairly thin but tough, purplish-red skin turning dark-purple or nearly black when ripe; smooth, glossy; enclosing very acid to fairly sweet, often bitter, juicy, red or pink, juicy pulp, exuding flecks of latex. There may be 2 to 8 small, flat, brown seeds.² Traditionally the plant is used as astringent, appetizer, antipyretic; lessen-thirst, biliousness and in diseases of the brain. Earlier studies have shown that various parts of *C. carandas* possesses various activities like cardiotoxic, anticonvulsant, histamine releasing, neuropharmacological and diuretics, antipyretic, anticancer and hepatoprotective etc.³ Its leaves decoction is used against fever, diarrhea and ear ache, whereas roots serve as a stomachic, vermifuge, remedy for itches and insect repellent.⁴ The aim of present study was to evaluate the phytochemical constituents, antinociceptive, anthelmintic and cytotoxic activities of methanol extract of leaves of *C. carandas* in experimental animal models with a view to provide a pharmacological justification for the use of plant leaves in the management of different types of diseases.

METHODS

Chemicals and drugs

The following drugs and chemicals used in the current study: Diclofenac-Na (Square Pharmaceuticals Ltd., Bangladesh), acetic acid (Merck, Germany), Albendazole (Square Pharmaceuticals Ltd., Bangladesh), DMF, methanol and DMSO (Merck, Germany), vincristine sulphate (Gedeon Richter), distilled water.

Plant materials and extraction

The leaves of *C. carandas* were collected from Dinajpur district, Bangladesh in the month of May, 2011. The plant was identified by the respective scientist of Bangladesh National Herbarium Institute, Mirpur, Dhaka. An accession number was given from there and a voucher specimen (DACB: 35587) has been deposited in the herbarium for future reference. The leaves were dried for one and half week in a shaded place. After drying, the plant part was grinded by blender machine (NOWAKE, JAPAN). Coarse powder was obtained after grinding.

Following the method of cold extraction, 100 g of powdered leaves of *C. carandas* was separately soaked in 550 ml of methanol for 15 days and then all the extracts were filtered through a cotton plug followed by Whatman filter paper number 1 and then concentrated by using a rotary evaporator at low temperature (40-50) °C and reduced pressure which provide greenish color extract (1.65 g).

Test animal

For the purpose of current study, swiss albino mice of both sex having 3-4 weeks of age, weighing between 20

to 25g were collected from the Animal Resources Branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B). Soft wood shavings were used as bedding of cages. Animals were maintained under standard environmental conditions: temperature (24.0±1.0°C), relative humidity (55-65% and 12 hrs. light /12 hrs. dark cycle). Husk and excreta were removed from the cages every day. The animals were fasted overnight before the experiments. Adult earthworm *Pheretima posthuma* were collected (due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human being) from moist soil. For anthelmintic study, all the worms were washed with normal saline to remove fecal matters. In brine shrimp lethality bioassay, brine shrimp nauplii are used as a favorable monitor for screening and fractionation in the discovery of new bioactive natural products.⁵

Phytochemical screening

The crude methanol extract of *C. carandas* leaves were qualitatively tested for detection of different phytochemical groups such as glycosides, alkaloids, steroids, flavonoids, tannins, saponins and reducing sugar by standard test procedures.⁶

Antinociceptive property

Antinociceptive means a drug that selectively relieves pain by acting in the CNS or on peripheral pain mechanisms, without significantly altering consciousness. So, antinociceptive activity means capacity of a substance to neutralize the pain sensation.⁷ Pain has been officially defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Pain acts as a warning signal against disturbances of the body and has a proactive function.⁸

Acetic acid-induced writhing test

Evaluation of antinociceptive effect was performed by acetic acid-induced writhing model in mice. The acetic acid-induced writhing method is an analgesic behavioral observation assessment method that demonstrates a noxious stimulation in mice. The test consists of injecting the 0.7% acetic acid solution intraperitoneally and then observing the animal for specific contraction of body referred as 'writhing'. A comparison of writhing was made with the positive control Diclofenac-Na. Control and test samples are given orally 30 min prior to acetic acid injection but Diclofenac-Na was administered intraperitoneally 15 min before injection of acetic acid. After an interval of 5 min, the mice were observed for specific contraction of body referred to as writhing for the next 10 min. If the sample possesses antinociceptive property, the animal that received the sample will give lower number of writhing than the control, i.e. the sample having analgesic activity will inhibit writhing.⁹

Anthelmintic activity

Fresh juice of the leaves of *C. carandas* was dissolved in minimum amount of DMF and the volume was adjusted to 10 ml with saline water. All drugs and juice solutions were freshly prepared before starting the experiment. In each case, 6 earthworms released into 10 ml of desired formulations as follows: vehicle (5% DMF in normal saline), Albendazole (25, 50 and 100 mg/ml) and fresh juice (25, 50 and 100 mg/ml) of the leaves of *C. carandas* in normal saline solution containing 5% DMF. Observation was made for the time taken to paralysis and death of individual worm. Paralysis was said to occur when the worms were not able to move even in saline solution.¹⁰

Cytotoxicity study

Brine shrimp lethality bioassay technique was applied for the determination of cytotoxic property of the plant extractive. Brine shrimp eggs collected from pet shops were used as the test organism. Seawater was taken in the small tank. Shrimp eggs were added to one side of the tank, and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and with the help of a pasteur pipette 10 living shrimps were added to each of the vials containing 5 ml of simulated seawater.

Preparation of positive control group

Vincristine sulphate was used as the positive control. 0.4 mg of vincristine sulphate was dissolved in 100 µL of DMSO to get an initial concentration of 40 µg/ml from which serial dilutions are made using DMSO to get 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 and 0.078 µg/ml, respectively. Then the positive control solutions are added to the remarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water.

Preparation of negative control group

30 µL of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii. If the brine shrimps in these vials show a rapid mortality, then the test is considered as invalid as the nauplii died due to some reasons other than the cytotoxicity of the compounds.

Preparation of test groups

4 mg of sample was dissolved in 100 µL of DMSO to get the stock solutions. Then 50 µL of solution was taken in each test tube containing 5 ml of simulated seawater and 10 shrimp nauplii. Thus final concentration of the prepared solution in the test tube was 400 µg/ml. Then a series of solutions of varying concentrations 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, and 0.781 µg/ml, respectively were prepared by serial dilution.

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration. The median lethal concentration (LC₅₀) of the test samples was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.¹¹

Statistical analysis

All experiments were performed thrice and the results averaged data were expressed as mean±SD.

RESULTS

Preliminary phytochemical screening revealed that, the methanol extract of the leaves of *C. carandas* contains alkaloids, steroids, flavonoids, tannins, saponins and reducing sugar which are presented in Table 1. In antinociceptive activity test, methanol extract of leaves showed inhibition of writhing of 55.59% and 67.10% at the dose of 200 and 400 mg/kg body weight, respectively (Table 2) that were comparable to the standard drug, Dichlofenac-Na (70.70% of inhibition).

From the results of the present study, it is clearly indicated that *C. carandas* produced anthelmintic activity by observing the time of paralysis and the time of death of earthworms *Pheretima posthuma* compared with the standard drug, Albendazole. The range of minimum to maximum time of paralysis and death of Albendazole at the conc. of 100 mg/ml was 3 min. 45 sec. to 10 min. 6 sec. whereas; the range of minimum to maximum time of paralysis and death of the fresh leaves juice was 4 min. 25 sec. to 7 min. 20 sec., respectively. It is evident from experimental data that the fresh juice of the leaves of *C. carandas* showed potent anthelmintic activity. Vehicle (5% DMF in normal saline) has no effect on both paralysis and death of individual worm. The results of anthelmintic activity of *C. carandas* are given in Table 3.

In brine shrimp lethality bioassay, the lethality of the methanol leaves extract of *C. carandas* to brine shrimp was determined and the summary expressed in Table 4. The leaves extract of *C. carandas* showed toxic activity against brine shrimp nauplii with LC₅₀ value of 4.52 µg/ml when compared with the standard, vincristine sulphate (0.777 µg/ml). In comparison with positive control (vincristine sulphate), the leaves extract signifies moderate cytotoxicity to brine shrimp nauplii. The LC₅₀ of the test samples after 24 hr. was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration (toxic concentration) and the best-fit line was obtained from data by means of regression analysis. The regression analysis data are given in the Table 5.

Table 1: Result of different phytochemical group test of methanol extract of leaves of *C. carandas*.

Sample	Glycosides	Alkaloids	Steroids	Flavonoids	Tannins	Saponins	Reducing sugar
Methanol extract	-	+	+	+	+	+	+

Here, + = Presence, - =Absence

Table 2: Effect of methanol leaves extract of *C. carandas* on acetic acid-induced writhing in mice.

Treatment	Dose (mg/kg b.w.)	Number of writhing	% of inhibition
Control (DMSO+water)	0.1 ml/Mice, oral	37.54 ±1.67	0.00
Diclofenac-Na	1.0, intraperitoneal	11.00 ±1.53	70.70
Group-I	200, oral	16.67 ±2.11	55.59
Group-II	400, oral	12.35 ±1.26	67.10

Values are expressed as mean ± SEM, where n = 5, Control: DMSO+Water (0.1 ml/Mouse), Positive control: Diclofenac-Na (1.0 mg/kg b.w.), Group I = *C. carandas* (200 mg/kg b.w.), Group II = *C. carandas* (400 mg/kg b.w.).

Table 3: *In vitro* anthelmintic activity of methanol extract of leaves of *C. carandas*.

Test Samples	Conc. (mg/ml)	Time taken for paralysis (minutes)	Time taken for death (minutes)
Albendazole	25	6 min 11 sec	13 min 16 sec
	50	4 min 23 sec	11 min 33 sec
	100	3 min 45 sec	10 min 6 sec
Fresh juice of leaves	25	7 min 5 sec	12 min 5 sec
	50	5 min 17 sec	11 min 26 sec
	100	4 min 25 sec	7 min 20 sec

Table 4: Effects of methanol extract of leaves of *C. carandas* on brine shrimp nauplii.

Methanol extract of the leaves				Vincristine Sulfate			
Conc. (C) (µg/ml)	Log C	% Mortality	LC ₅₀ (µg/ml)	Conc. (C) (µg/ml)	Log C	% Mortality	LC ₅₀ (µg/ml)
400	2.602	100	4.52	40	1.602	100	0.777
200	2.301	90		20	1.301	90	
100	2	90		10	1.000	90	
50	1.699	80		5	0.698	80	
25	1.397	70		2.5	0.397	70	
12.5	1.097	60		1.25	0.096	50	
6.25	0.796	60		0.625	-0.204	40	
3.125	0.495	50		0.312	-0.505	40	
1.563	0.194	40		0.156	-0.806	30	
0.781	-0.107	20		0.078	-1.107	20	

Table 5: Regression analysis data for vincristine sulphate and methanolic extract of leaves of *C. carandas*.

Sample	LC ₅₀ (µg/ml)	Regression equation	R ²
Vincristine sulphate (Positive control)	0.777	y=30.80x + 53.38	0.973
Methanol extract of <i>C. carandas</i>	4.52	y=26.98x + 32.34	0.964

DISCUSSION

Phytochemical study of methanol leaves extract of *C. carandas* was performed by using preliminary phytochemical group tests. From the study, it was found several important phytochemical groups like alkaloids, steroids, flavonoids, tannins, saponins and reducing sugar. It has been reported that different phytochemicals

are responsible for antibacterial, cytotoxic, antinociceptive and neuropharmacological activities.¹² In case of acetic acid-induced writhing test, a dose dependent reduction in the number of abdominal constriction was observed in animals treated with different concentration of methanol leaves extract of *C. carandas* at the doses of 200 and 400 mg/kg b.w., inhibition of writhing response was observed 55.59% and

67.10%, respectively that was comparable to standard drug, Dicholofenac-Na (70.70% of inhibition). From the data, we observed that methanol leaves extract significantly inhibited writhing response induced by acetic acid in a dose dependent manner. Reducing sugar and flavonoids may be responsible for the antinociceptive activity of *C. carandas* leaves extract.^{13,14}

It is evident from experimental data that the fresh juice of the leaves of *C. carandas* showed potent anthelmintic activity. Results were comparable with standard drug, Albendazole. Preliminary phytochemical screening of methanol leaves extract of *C. carandas* showed the presence of steroids, flavonoids and tannins. The methanol leaves extract possess potent anthelmintic activity due to the availability of those important phytochemical groups. The anthelmintic activity has already been reported by Mishra et al.¹⁵

In case of cytotoxicity studies, the plant extract of *C. carandas* showed significant cytotoxic activity against brine shrimp nauplii with LC₅₀ value, 4.52 µg/ml when compared with the standard, vincristine sulfate (0.777 µg/ml). In comparison with standard methanol extract of leaves possesses moderate cytotoxic activity. The cytotoxic effect of plant is due to the presence of secondary metabolites like alkaloids, steroids and flavonoids which are present in the methanol extract of leaves of *C. carandas*.¹⁶

CONCLUSION

Plants are considered as one of the most important and interesting subjects that should be explored for the discovery and development of newer and safer drug candidates. From all the conducted experiments, it can be concluded that the methanol extract of leaves of *C. carandas* showed potent antinociceptive and anthelmintic activities and possesses moderate cytotoxic activity. Dose-dependent activity was also identified by all the performed pharmacological investigations. To sum up, these findings together demonstrate that *C. carandas* is an excellent plant candidate for further investigations of new bioactive compounds. Therefore, in depth extensive study should be an urgency to sort out their activities against various diseases and to isolate bioactive principles for novel leads compounds for new drug development.

ACKNOWLEDGEMENTS

The author is grateful to the Department of Pharmacy, Stamford University Bangladesh, Dhaka-1217 for providing all supports to carry out of this research.

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: The study was approved by the institutional ethics committee

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Cite this article as: Parvin MN. Phytochemical screening, antinociceptive, anthelmintic and cytotoxicity studies of the leaves of *Carissa carandas* Linn. (Family: Apocynaceae). Int J Sci Rep 2018;4(5):119-23.