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Cardiotonic potential of *Carissa opaca* Stapf ex Haines: A study on isolated rabbit heart

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Abstract: *Carissa opaca* (C.O) is a wild shrub, belonging to the family Apocynaceae. The medicinal virtues of this plant have long been known. The present study demonstrates the effects of aqueous-methanolic extract and various fractions (n-butanolic and aqueous) of *Carissa opaca* on cardiovascular parameters. The perfusion pressure (PP), force of contraction (FC) and heart rate (HR) were assessed on isolated heart of rabbit using Langendroff's technique for crude extract and fractions of C.O, followed by the elucidation of the mechanism of action after estimating toxicity of the plant. Negative ionotropic and positive chronotropic effects, with an increase in PP in isolated perfused rabbit heart were observed the with plant extract and fractions. The aqueous-methanolic extract exhibited maximum response at 1mg/ml while the n-butanolic and aqueous fractions showed a maximum response at 1mg/ml and 10µg/ml respectively. Both fractions produced the same response when treated with atropine (10^{-5} M), however the actions of adrenaline (10^{-5} M) and calcium chloride (10^{-5} M) remained unblocked. Acute toxicity studies indicated that the plant was safe up to 2000 mg/kg and sub-chronic studies demonstrated that no significant change in haematological and biochemical parameters observed. In conclusion, this study supports the folkloric claim of C.O extract.

Keywords: *Carissa opaca*, isolated rabbit heart, cardiotonic, toxicity study.

INTRODUCTION

There is a continuing increase in the demand and use of natural plants and traditional medicines, far and wide, for the management of various ailments. The reasons for which may be attributed to the growing population, high costs and poor availability of medication and treatments, and the occurrence of adverse effects and drug resistance. Thus, despite new advancements and improvements in medicine, a major portion of the world's population relies on natural remedies over synthetic products (Ekor, 2014). According to the World Health Organization (WHO) natural products could satisfy the medicinal needs of the majority world's population (Yuan *et al*., 2016). At the beginning of 21th century, 11% of 252 basic and essential drugs, enlisted by WHO were of plant origin. According to a study, during the last three decades, upto 50% drugs used worldwide are either directly or indirectly of plant nature (Thomford *et al*., 2018).

Carissa opaca Stapf ex Haines locally called as Karaunda, Jungli Karonda (Hindi), Karvand (Marathi), Karamdika (Oriya) belongs to family Apocynaceae and is distributed widely in Pakistan (Punjab-Himalayas), India, Burma and Sri Lanka (Ajaib *et al.,* 2010). Traditionally, *Carissa opaca* has been used to combat several ailments and conditions; including eye disorders, fevers, jaundice, hepatitis, wound healing, for the treatment of asthma and as a stimulant. The fruit of said plant combined with roots of *Mimosa pudica* have been used as aphrodisiac and the plant has also been used as a fly repellent (Ahmad *et al.,* 2009). The plant is known to have cardiotonic action (Abbasi *et al.,* 2009). Analysis of different parts of plant (fruits, seeds, and leaves) showed that it is rich in potassium, magnesium, iron, zinc, copper, and chromium (Khan *et al.,* 2010). C.O is reported for having antioxidant capabilities (Sahreen *et al.,* 2013a), antibacterial activity and anticancer properties (Sahreen *et al.,* 2013b), nephro and hepato-protective potential (Sahreen *et al.,* 2014a) and pulmonary fibrosis (Sahreen *et al.,* 2014b). Based on the traditional claim of C.O*,* the present study was planned for the pharmacological evaluation of cardiovascular ailment from the extract of leaves of C.O in rabbit isolated heart model after estimating of the toxicity profile.

MATERIALS AND METHODS

Chemicals used

Methanol, Sodium chloide, calcium chloride and glucose (Sigma Aldrich, USA), bicarbonate (BDH laboratory supplies poole, Bh 15 ITD, England), potassium chloride (Riedel-de-Harn AGD-3016, Seelze Germany), Di-hydrogen sodium phosphate (Merck KGaA, 64271 Darmstadt, Germany), magnesium sulphate (Panreac Quimica SA E-08110 Montcada Reizac Espana),

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Carbogen (95% oxygen and 5% carbon dioxide), adrenaline (Elite pharma) and atropine (BDH Chemicals Ltd., Poole, England) were used.

Experimental animals

Sprague-Dawley rats (180-250g), Swiss Albino mice (20-40g) and rabbits (1000-1500g) were used. The animals were housed in a carefully maintained environment (23-25°C) at the animal house provided by Department of Pharmacy, University of Sargodha, Sargodha, Pakistan. All animals were fed standard diet and given free access to tap water *ad labitum*, and treated in accordance with standard procedures of the National Research Council.

Preparation of plant extract and fractions

Leaves of *C.O* were cleaned with tap water, dried in shade and comminuted to a fine powder form. Extraction was carried out using cold maceration process. N-butanolic and aqueous fraction were obtained using the Successive solvent extraction method with activity-directed fractionation of the crude plant extract. After fractionation, samples were concentrated in rotary evaporator, dried in oven and stored in a refrigerator until used for analysis (Adeshina *et al.,* 2012)[14].

Experimental procedures

Acute toxicicity test of C.O in mice

Male albino mice, weighing 20-40 g, were randomly assorted into five groups (n= 6). Group I was assigned as control and given normal saline (10ml/kg) only, while the remaining groups (2, 3, 4 and 5) were each given the plant extract in various concentrations i.e. 25, 50, 100 and 250 mg/kg respectively. Any change in general behavior was observed for 5 hours followed by observation for mortality during the next 24 hours. Due to the absence of mortality, another five groups were taken, and exempting control, treated with higher doses of the crude extract i.e. 500, 1000, 1500, 2000 mg/kg respectively. All doses were given orally to animals had been subjected to overnight fasting before experimentation. (Shetty *et al.,* 2007; Falya *et al*., 2020).

Sub-chronic toxicity test of Carissa opaca (C.O) in rats

Male rats weighing 180-250 g were divided in 2 groups (n=5). The first group was made control and given normal saline, while selected dose of crude extract of *C. O* (200 mg/kg) were administered to the second group daily p.o. for 28 days. Any changes in general behavior, body weight, food and water intake, discharge and tremors were observed daily. On the 29th day, animals were anaesthetized with thiopental sodium, a cardiac puncture was done on overnight fasted animals, to collect blood for the estimation of biochemical and hematological parameters, additionally the organs were collected and weighed individually (Biswas *et al.,* 2010).

Effect of C.O aqueous-methanolic extract on PP, FC and HR on isolated perfused rabbit heart

The experiment was conducted in accordance with the method prescribed by Langendorff, 1895 with some modifications (Langendorff, 1895). Each rabbit was injected with heparin (i.p) 30 min prior to dissection. Heart with 1cm intact aorta was dissected out of the animal and immediately transferred to Krebs-Henseleit solution to remove excess blood in the heart, which was then mounted on a modified Langendorff apparatus. The aorta was fixed to a glass cannula, connected with a pressure transducer. At first the perfusion fluid was run through the heart with an increased rate followed by maintenance at a steady rate. Clip, attached to the force transducer, was inserted at the apex of heart. Each of the transducer was connected with the PowerLab, and Lab Chart 5.0 software was used to record the readings. The force of contraction was adjusted at 2-4 g (Bell *et al.,* 2011). A stabilization period was given, after which the heart was made to function under normal conditions, during which the perfusion and heart rates were measured, as control. Thereafter the different prepared concentrations of the crude extract (10ng, 100ng, 1µg, 10 µg, 100µg and 1mg) were injected and changes in the PP, FC and HR were assessed. Fixed volume (5ml) of drug was injected via a three-way port, after. Prior to injection, each dose was filtered with a micro syringe filter. The changes in the values PP, FC and HR were expressed as % change for control.

$$\text{Percentage change} = \frac{T - C}{C} \times 100$$

Where, C = control reading, T = tested drug reading.

Simialry, the actions of aqueous fraction of *Carissa opaca* (ACO) and butanolic fraction of *Carissa opaca* (BCO) and on PP, FC and HR were assessed following the same method as described above.

Effect of C.O fractions on PP, FC and HR in the presence of atropine (10^{-5} M), adrenaline (10^{-5} M) and calcium Chloride (10^{-5} M)

Rabbit heart was mounted and stabilized on Langendorff's apparatus as explained before. Selected most significant concentration of ACO (10µg) and BCO (1mg/ml) were injected and after giving a washout of 4-5 minutes, same doses were repeated with the administration of atropine (10^{-5}M). Changes in heart rate, force of contraction, and perfusion pressure were recorded. These parameters were also determined with and without the presence of adrenaline (10^{-5} M) and calcium chloride (10^{-5} M) (Alamgeer *et al*, 2016).

Ethical approval

All experimental procedures were approved (No. IAEC/UOS/2016/46) by the Ethical Committee of College of Pharmacy, University of Sargodha, Pakistan.

STATISTICAL ANALYSIS

All results obtained are expressed as Mean ± Standard error of mean (S.E.M). Using GraphPad Prism Software, version 8, student's t-test and two way ANOVA followed Bonferroni's test were applied, taking the value of $p<$ 0.05 as significant.

RESULTS

Acute toxic effects of C.O in mice

C.O was found to be safe at 2000 mg/kg body weight, no toxicity was observed at this dose.

Sub-Chronic effects of C.O in rats

Sub-chronic administration of C.O in rats showed no toxicity, after administration of 200mg/kg for 28 days. The general behavior and body weight remained unaltered. Additionally, signs of toxic effects were also absent in hematological and biochemical parameters (tables 1-4).

Effects of C.O crude extract on force of contraction, heart rate and perfusion pressure in isolated rabbit perfused heart

In isolated heart preparation, C.O produced a decrease in FC while significant increase was observed in the PP and HR, in a concentration-dependent manner at all concentrations. The maximum response in all the three parameters was observed at 1mg/ml as elucidated in table 5.

Effects of ACO and BCO on force of contraction, heart rate and perfusion pressure in isolated rabbit heart

A similar pattern was observed while estimating the effects of butanolic and aqueous fractions of the *C. opaca* on cardiac parameters, aqueous fraction being more

Table 1: Assessment of the effect of *Carissa opaca* on complete blood count in rats

Hematological parameters	Control	Treated
WBC X1000	15.3 ± 0.37	16.0 ± 0.58^{ns}
RBC (10^6/mm^3)	8.32 ± 0.23	8.68 ± 0.27^{ns}
Hb (g/dl)	14.0 ± 0.55	14.3 ± 0.72^{ns}
HCT (PCV %)	48.99 ± 0.86	49.7 ± 0.88^{ns}
MCV (fl)	65.0 ± 1.94	65.7± 1.76^{ns}
Neutrophils %	11.6 ± 0.30	11.4 ± 0.41^{ns}
Lymphocyte %	76.8 ± 2.68	77.3±2.40^{ns}
Monocytes %	4.30 ± 0.57	4.47±0.29^{ns}
Platelets X1000	1220 ± 34.2	1220± 37.50^{ns}

Results are expressed as means ± S.E.M (n = 3), where ns = non-significant vs control

Table 2: Effect of *Carissa opaca* leaves on Lipid profile in rats

Lipid profile (mg/dl)	Control	Treated
Triglycerides	122±1.45	120 ± 1.03^{ns}
Total cholesterol	124 ± 2.06	127± 1.20^{ns}
HDL	48.7±1.76	46.7 ± 2.44^{ns}
LDL	95.6 ± 1.37	96.7 ± 2.40^{ns}
VLDL	24.5 ± 2.48	25.7±2.03^{ns}

Results expressed as means ± S.E.M, where ns = non-significant vs control

Table 3: Effect of *Carissa opaca* on serum marker enzymes and proteins in rats

Liver function tests	Control	Treated
Bilirubin (mg/dl)	0.47±0.09	0.50± 0.06^{ns}
ALT (U/L)	35.7±2.03	35.1±2.73^{ns}
AST (U/L)	99.7±0.88	99.5±0.54^{ns}
ALK (U/L)	47.7±0.89	48.0±1.44^{ns}
Albumin (g/dl)	4.63±0.08	4.36±0.19^{ns}

Results are expressed as means ± S.E.M (n = 3), where ns = non-significant vs control group.

Table 4: Estimation of effect of *Carissa opaca* on various organs weights in rats

Weights of organs (g)	Control	Treated
Heart	0.47 ± 0.02	0.46 ±0.03^{ns}
Liver	6.57 ± 0.18	6.51±0.17^{ns}
Kidney	2.83± 0.15	2.87 ± 0.15^{ns}

Results are expressed as means ± S.E.M (n = 3), where ns = non-significant vs control group

Table 5: Effect of C.O on force of contraction, heart rate and perfusion pressure of isolated rabbit heart

Conc.	Perfusion pressure (mmHg)			Force of contraction (g)			Heart rate (beats/min)		
	Control	Treated	% Change	Control	Treated	% Change	Control	Treated	% Change
10ng	63.3±0.65	81.6±0.81***	28.91	3.4±0.11	2.44±0.21***	-28.45	108±4.59	115±1.04**	6.48
100ng	67.7±1.12	82.6±0.78***	22.01	3.41±0.04	2.33±0.13***	-31.67	106±0.84	136 ±1.46***	28.30
1µg	62.7±0.16	82.6±0.19**	31.74	2.91±0.03	2.31±0.04**	-20.62	104±1.83	136± 2.92***	30.76
10µg	61.6±0.83	86.4±0.88**	40.26	3.10±0.04	2.26±0.07***	-27.10	103±1.61	137±1.63***	33.01
100µg	64.7±1.45	87.5±1.60***	35.23	3.02±0.11	2.23±0.11***	-26.16	104±1.02	138±1.98***	32.69
1mg/ml	63.9±1.05	90.4±1.32***	41.47	3.09±0.10	2.15±0.03***	-30.42	84.7±4.37	139± 2.87***	64.11

Values are expressed as means ± S.E.M (n = 6), where ** =p<0.001 and *** =p<0.0001 significant when compared to the control.

Table 6: Effect of ACO on force of contraction, heart rate and perfusion pressure of isolated rabbit heart

Doses	Perfusion pressure(mmHg)			Force of contraction (g)			Heart rate (beats/min)		
	Control	Treated	% Change	Control	Treated	% Change	Control	Treated	% Change
10ng	59.2±1.73	66.3±1.86*	11.9	3.23±0.126	2.62±0.130**	-18.89	101±1.76	120 ±1.15***	18.81
100ng	56.1±0.86	67.0±2.52*	19.5	3.21±0.107	2.66±0.0208***	-17.14	105±2.91	121±2.03***	15.23
1µg	57±1.76	68.7±2.03*	20.5	3.22±0.0186	2.53±0.0333***	-21.46	104±3.06	125±2.60**	20.19
10µg	54.5±2.31	77.2±1.15**	41.65	3.11±0.123	2.28±0.120***	-26.69	86.0±3.06	129±4.58**	50
100µg	55.8±1.15	75.3±2.40**	34.9	3.23±0.182	2.39±0.0769**	26.01	101±2.67	132±4.41**	-30.69
1mg/ml	57.1±2.60	78.7±0.667**	37.8	3.33±0.119	2.45±0.125**	26.43	97.7±1.45	130±0.882***	-33.06

Table 7: Effect of BCO on force of contraction, heart rate and perfusion pressure of isolated rabbit hearts

Conc.	Perfusion pressure (mmHg)			Force of contraction (g)			Heart rate (beats/min)		
	Control	Treated	% Change	Control	Treated	% Change	Control	Treated	% Change
10ng	61.2±1.02	81.8±0.86***	33.66	3.58±0.03	2.46±0.17**	-31.29	112±1.04	125±0.12**	11.60
100ng	63.3±1.24	84.3±1.26***	33.17	3.51±0.11	2.42±0.16**	-31.05	108±2.58	133± 3.36**	23.14
1µg	63.2±1.54	86.0±1.18***	36.07	3.54±0.24	2.62±0.06*	-25.98	109±2.29	134±1.63***	22.93
10µg	64.0±0.84	86.8±0.89***	35.62	3.28±0.18	2.45±0.04*	-25.30	105±1.85	138±1.38***	31.42
100µg	64.7±1.45	88.0±1.24***	36.01	3.15±0.15	2.32±0.02**	-26.34	106±0.99	139±0.13***	31.13
1mg/ml	65.7±1.12	91.9±0.51***	39.87	3.10±0.11	2.20±0.10**	-29.03	96±4.37	139±0.76***	44.79

Values are expressed as means ± S.E.M (n = 3), where * = p<0.05 ** = p<0.001 and *** = p<0.0001 significant vs control.

potent. Both fractions exhibited a significant decrease in force of contraction. However PP and HR were increased in a concentration-dependent manner at all concentrations. The maximum response in PP, FC and HR was observed at 10µg/ml for ACO and 1mg/ml for BCO (tables 6, 7).

Effect of ACO and BCO on force of contraction, heart rate and perfusion pressure in the presence of atropine (10^{-5} M)

ACO at 10µg/ml significantly decreased the FC and increased HR and PP. Negative ionotropic and positive chronotropic effects produced by ACO and 1mg/ml of BCO were not inhibited when given with atropine 10^{-5} M (fig. 1 A,B).

Effect of Adrenaline (10^{-5} M) on force of contraction, heart rate and perfusion pressure in the presence of ACO and BCO

Adrenaline (10^{-5} M) produced a significant increase in PP, FC and HR of isolated rabbit heart, both in absence and presence of ACO (1µg/ml) and BCO (1mg/ml) as shown in (fig. 1 C,D).

Effect of calcium chloride (10^{-5} M) on force of contraction, heart rate and perfusion pressure in the presence ACO and BCO

Calcium chloride (10^{-5} M) displayed a significant increase in FC, HR and PP of isolated rabbit heart. The positive ionotropic and chronotropic effects of calcium chloride (10^{-5}M) were not inhibited on treatment with ACO. Similar effects were observed when $CaCl_2$ (10^{-5} M) was injected in the presence of BCO (fig. 1 E,F).

DISCUSSION

The frame of prevailing ethno-medical knowledge has directed to prodigious advances in health care system (Yuan *et al*., 2016). Medicinal plants have been acknowledged for their effects on different parameters of the cardiovascular system. In screening the pharmacological activity of plant products, it is essential to initially assess and evaluate, any possible toxic characteristics. The observations made from the acute toxicity study could aid in classifying and provide preliminary information about the mode of toxic action of a substance, calculating the dose for experimental animal

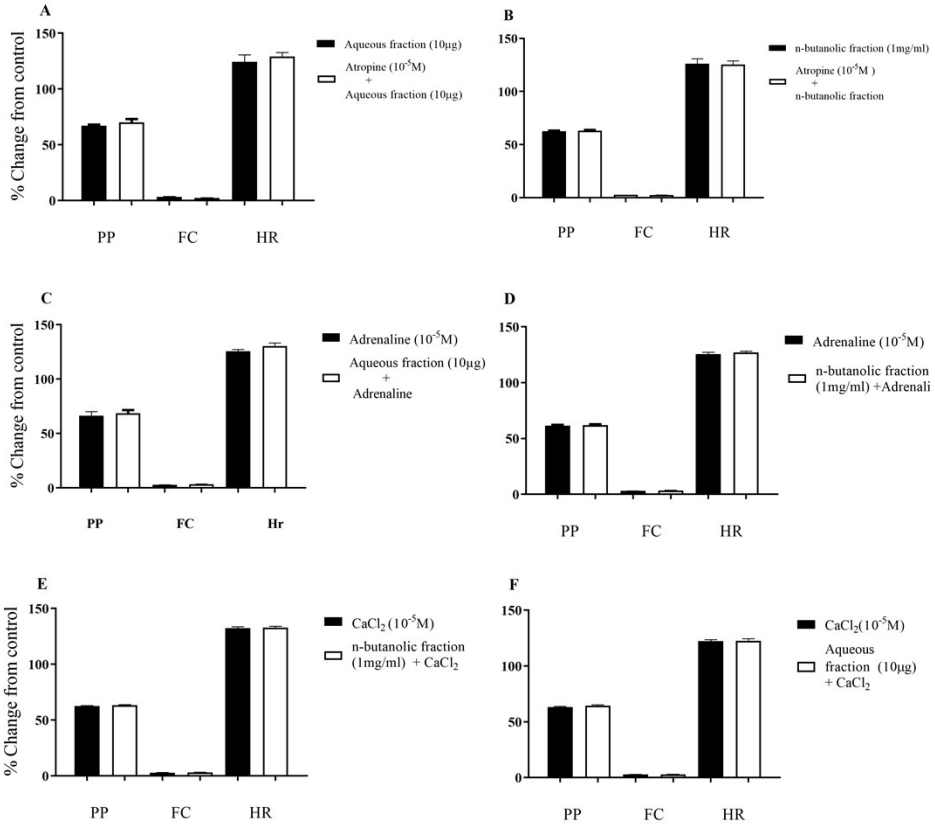

Fig. 1: The bar graphs shows the effects of atropine 10^{-5} M (A, B), adrenaline 10^{-5} M (C, D) and calcium chloride 10^{-5} M (E, F) in presence and absence of n-butanolic and aqueous fractions of *Carissa opaca* on perfusion pressure (PP), force of contraction (FC) and heart rate (HR) of isolated rabbit heart (n=6).

studies and determination of LD_{50} values, which pave the road to evaluation of potential drug activity (Ukwuani *et al.,* 2012). Till date, chemical labeling and the classification of acute systemic toxicity is done as recommended by the Organisation for Economic Co-operation and Development (OECD), according to the oral LD_{50} values, the classes being; 1) Very toxic, <5 mg/kg body weight; 2) Toxic, >5 <50mg/kg; 3) Harmful, >50 <500mg/kg; 4) No label, >500 <2000mg/kg and 5) Least toxic, >5000mg/kg (Parasuraman, 2011). In the present study, 2000 mg/kg of the C.O extract did not show any adverse effect, falling under OECD class 4 status (LD_{50} >500 <2000mg/kg). On this basis, one tenth (200 mg/kg) of the maximum dose tested (2000 mg/kg), was selected for evaluation of further study. Sub-chronic administration specifically provide info regarding toxicity in target organs and identify the level of adverse effects that are non-observable (National Research Council, 2006). Also they can be used in the determination of dose regimens for longer-term studies. In sub-chronic study the administration of the C.O extract for 28 days produce no significant signs of any mortality or toxicity were detected. It has been observed that with higher doses, crude plant extracts may be converted into a toxic metabolite that could interfere with gastric function and in turn decrease the efficiency of food conversion. (Chokshi, 2007), however no such effects was observed after sub-chronic study, thus the plant may be considered safe.

In isolated perfused rabbit heart, C.O and their fractions significantly decreased the FC and increased the HR and PP at most of the concentrations used. The involvement of muscarinic receptors for ACO and BCO were ruled out, as the negative ionotropic and positive chronotropic effects and the increase in PP remained unaffected when treated with atropine. B-adrenergic blockers and calcium channel blockers also decrease heart contractility (Magnussen & Kudsk, 2009). Adrenaline was also observed to increase the PP, FC and HR. However, in the presence of ACO and BCO there was no change in the effects of adrenaline, ruling out the involvement of β-adrenergic receptors. The action of both fractions on calcium channels were observed with calcium chloride ($CaCl_2$). Calcium chloride (10^{-5} M), increased the PP, FC and HR, however the fractions did not block the effect of calcium chloride. So the current study was unable to identify the involvement of exact underlying mechanism. The other possible targets are potassium channels and nitric oxide-dependent coronary vasodilation. Moreover, the C.O extract is enriched with phytochemical

constituents i.e. flavanoids, anthraquinones, cardiac glycosides, coumarins, alkaloids, terpenoids, saponins and tannins (Sahreen *et al.,* 2013b). Thus, to trace the underlying mechanism(s) the mentioned phytochemicals can also be investigated to elucidate the negative ionotropic and positive chronotropic effects of C.O in isolated rabbit heart.

CONCLUSION

It is concluded from the study that various phytochemicals constituents in the C.O extract/fractions are expected to responsible for negative inotropic and positive chronotropic effects on isolated rabbit`s heart. However, further studies are required to isolate these pharmacologically active phytochemical constituents and elucidate their exact mechanism of action.

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