



## Therapeutic potential of *Ceratonia siliqua* extract for the management of asthma and hypertension

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### ABSTRACT

The current work was performed to explore the pharmacological mechanisms involved in the management of asthma and hypertension along with the safety profile of the *Ceratonia siliqua* (*C. siliqua*/Carob) pods. The bronchorelaxant, vasorelaxant, and cardioselective activities of *C. siliqua* pods were investigated using isolated rabbit tracheal, aortic, and paired atrial fragments on the Power lab data acquisition system. Normotensive rats were used to study antihypertensive activity. The plant extract and its fractions relaxed the carbachol-induced contraction in the tracheal fragments and shifted the concentration-response curve of carbachol towards the right confirming the muscarinic receptor antagonist activity. The relaxation of phenylephrine-induced contraction in an aortic fragment by the extract showed  $\alpha$ -adrenergic blocking activity. Furthermore, the extract produced a cardio-selective response in the paired atria and decreased the blood pressure in anesthetized normotensive rats. The plant extract proved to be non-toxic in oral acute and chronic toxicity studies and did not demonstrate any sign of histopathological lesions. These results suggested that the plant extract was non-toxic and could be used in the management of lifetime therapies of respiratory and cardiovascular disorders without any unwanted effects.

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### Introduction

The function of the respiratory system is normally mediated through the contraction and relaxation of respiratory smooth muscles. The smooth muscles of the airway are regulated by the multiple control systems i.e. (i) mediators that alter airway tone by dilator and constrictor action (ii) secretory glands and neuronal system (iii) reflexes by parasympathetic (cholinergic) innervations to the smooth muscle (1). The disturbance in the respiratory functions leads to multiple chronic respiratory disorders including cough, shortness of breath, asthma, and chronic obstructive pulmonary diseases (COPD) (2). Microorganisms, such as bacteria, viruses, and fungi, along with their microbiome exist throughout the

respiratory tract. Most microbiological knowledge of the respiratory tract relates to bacteria, however, the pathogenic effects of the viruses including respiratory syncytial virus, influenza A virus, and rhinovirus, have widely been studied. Metagenomic approaches are identifying many new viruses in the respiratory tract with unknown effects, including a novel polyomavirus and novel coronavirus (Covid-19) (3). The mediators released from the cells initiate the pronounced hypertrophic and hyperplastic responses in bronchial smooth muscles as well as the mucus-secreting glands. The production of an excessive amount of mucus may be problematic as it can lead to choking, edema, bronchoconstriction, and impairment of muco-ciliary activities of the respiratory tract (4).

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Hypertension is a major risk factor for cardiovascular diseases. At present, almost 1 billion persons are affected by hypertension throughout the world. The national health survey of Pakistan assessed that hypertension affects 33% of adults over the age of 45 years(5). Multiple pathophysiological mechanisms are known to be contributing to hypertension. The most prevalent mechanisms include; (i) enhanced functioning in the autonomic sympathetic nervous system (ii) enhanced functioning in the rennin-angiotensin-aldosterone system (iii) decreased functioning of the vasodilatation mechanisms, e.g., synthesis and release of NO, and (vi) enhanced stiffness and decreased elasticity in the vascular system. The determinant factors of hypertension are genetic, demographic, and environmental including response to psychosocial stress, intake of excessive dietary sodium, and inadequate intake of potassium and calcium. Various metabolic disorders such as diabetes mellitus, insulin resistance, and obesity also may cause an increase in blood pressure by the production of vasoactive adipocytokines, which in turn play an important role in vasoconstriction, endothelial dysfunction, inflammation and increased oxidative stress(6). The role assigned to the natural products in the field of drug research and new drug development is very crucial as plant-based drugs have been used in developed countries to manage or prevent several hardly curable disorders like respiratory and cardiovascular diseases (7).

*Ceratonia siliqua* L. (Fabaceae) is also known as Kharnub/ Carob. Pods of *C. siliqua* are widely used in the food industry as a source of food supplements and additives. These are also used as a drug carrier in multiple formulations of pharmaceutical and nutraceutical products. These are a good source of carbohydrates, dietary fiber, fat, and protein. They also contain polyphenols, flavonoids, glucose, as well as sucrose. In addition to minerals like potassium, calcium, magnesium, iron, zinc, copper and amino acids (8). It has chocolate and coca-like flavour. It does not involve CNS stimulation or kidney stone formation because it is deficient in caffeine, theobromine, and oxalic acid. Locust bean gum (LBG) extracted from seed endosperm of pods is a white to creamy powder used as a versatile food additive approved by European Codex (9, 10). In Arabic countries, the carob fruit is used for preparing

popular beverages and traditional medicine to treat diabetes (11). Several pharmacological studies showed its antibacterial, anti-diabetic, anti-oxidant, anti-proliferative, antifungal, anti-atherosclerotic, cytotoxic, neuro-protective, gastro-protective and hepato-protective potential. *C. siliqua* pods are traditionally used in the treatment of diarrhea, vomiting, asthma, cough, hyper-peristalsis, cardiovascular diseases, high cholesterol, diabetes, obesity, pain, and inflammation (12).

As the *C. siliqua* pods possess folkloric repute to be effective in the management of respiratory and cardiovascular ailments; hence, the present study was undertaken to validate its status through the provision of a scientific basis for its use in native systems of medicines.

## Materials and methods

### Chemicals and reagents

All chemicals were procured from reputed vendors in Pakistan. Acetylcholine (Ach), atropine, carbachol (CCh), dicyclomine, phenylephrine (P.E), ethylenediaminetetraacetic acid (EDTA), magnesium chloride (MgCl<sub>2</sub>) and potassium chloride (KCl) were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Ketamine was acquired from Akhai pharma (Pvt). Ltd (Karachi, Pakistan). The organic solvents including methanol (MeOH) and dichloromethane (DCM) were acquired from VWR international (Pennsylvania, USA). Glucose, calcium chloride (CaCl<sub>2</sub>), magnesium sulphate (MgSO<sub>4</sub>), potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), sodium dihydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub>) and sodium bicarbonate (NaHCO<sub>3</sub>) was purchased from Carl Roth GmbH & Co. (Karlsruhe, Germany). Adrenaline and diazepam were obtained from the PDH pharmaceuticals (Lahore, Pakistan) and Roche pharmaceuticals (Karachi, Pakistan) respectively. Moreover, all the solutions were freshly prepared and sterile filtered before the experiments.

### Animals

Sprague-Dawley albino rats (♂/♀) weighing 150-210 g with an age of 3-4 months, Bulb/C mice (♂/♀) weighing 20-30 g with an age of 1.5-2 months, and local breed rabbits (♂/♀) having an average body weight of 1.2-1.7 kg (5-6 months old) were placed separately in steel cages filled with wood dust, under

controlled temperature ( $25\pm 3^{\circ}\text{C}$ ) and other environmental conditions (moisture: 60% and alternative 12 h light/dark cycle) in the animal house at Bahauddin Zakariya University, Multan, Pakistan. The animals were maintained on tap water and commercial animal feed *ad libitum* (Hi-Tech Feeds Pvt. Ltd. Lahore). Prior to the experiments, the animals were deprived of food for 12 h period during which drinking water was permitted. Appropriate care was exercised to minimize the number of animal use as defined by the 3R principle i.e., Replacement, Reduction, and Refinement. This study was carried out in strict compliance with the recommendations of the Institute of Laboratory Animal Resources (13). The experimental protocol was approved by the Animal Ethics Committee of Bahauddin Zakariya University, Multan, Pakistan (Protocol Number: EC/05PhDL/2013). The surgery in the chronic toxicity studies was performed under ketamine anesthesia. All animal handling was performed by the professionally-trained staff.

### Collection and identification

The fresh pods of *C. siliqua* Linn were collected from suburban locations of Mansehra, Pakistan. A renowned botanist (Dr. Abdul. Rehman. Niazi) from The University of the Punjab, Lahore, Pakistan was requested regarding authentication of the plant materials, and the representative sample with identification # 252015 was submitted in the departmental herbarium. Plant material was shade dried and converted into coarse powder through a purposeful grinding device.

### Extraction and fractionations

The hydro-methanol extract of pods (30:70) was prepared according to standard technique under reduced pressure at room temperature ( $27\text{-}30^{\circ}\text{C}$ ) employing a rotary evaporator. The approximate yield of pods extract was found to be 12.90 %. The crude extracts of pods of *C. siliqua*, Cr.Cf (10 g) was rendered soluble in distilled  $\text{H}_2\text{O}$  and was subjected to liquid-liquid fractionation with dichloromethane (DCM) to fractionate it into the DCM and aqueous fractions using standard procedures. The yields of dichloromethane (Dc.Cf) and aqueous (Aq.Cf) fractions were achieved on fractionation of Cr.Cf that was found to be 60 % and 40 % respectively (11).

### Isolation of alkaloid concentrates

The DCM fraction of the pods (Dc.Cf) contained alkaloids, resins, fats, and waxes. It was subjected to further extraction with acidified water (pH: 2-3; dilute HCl). As the alkaloids on acid treatment are converted to salt form; hence, they are more soluble in water and extracted in the aqueous phase. The aqueous acidic layer was rendered alkaline on treatment with sodium bicarbonate ( $\text{NaHCO}_3$ ) adjusting the pH to 10. In alkaline media, the alkaloids were changed to free alkaloid bases. These alkaloid bases were then extracted into the DCM phase. This phase became rich in alkaloids-like constituents and was designated as Dc.Af.

### Phytochemical analysis

Secondary metabolites of diverse chemical nature including alkaloids, flavonoids, phenols, saponins, and tannins were detected from the extract by reported procedures as described by Hamid et al(12). The phytochemical screening of the dichloromethane fraction of extract indicated the presence of alkaloids, resins, fats, and waxes (14).

### Ex-vivo experiments

#### Tracheal isolates of the rabbits

The rabbits ( $n=5$ ) were sacrificed by a blow on the back of the head, and the tracheal tube was removed from which a piece of the 3-4 mm ring was separated. A cut was made longitudinally on the ventral side to open the ring just opposite to the smooth muscle layer. The tracheal strip consists of smooth muscles present in the middle of the cartilaginous tissues. These tracheal isolates of rabbits were loaded into a tissue organ bath containing 20 mL Krebs's solution ( $37^{\circ}\text{C}$ ) and were continuously bubbled with a carbogen gas ( $\text{O}_2$ :  $\text{CO}_2$  - 95:5) mixture. The Krebs's solution comprised of NaCl (118.2 mM),  $\text{NaHCO}_3$  (25.0 mM),  $\text{CaCl}_2$  (2.5 mM), KCl (4.7 mM),  $\text{KH}_2\text{PO}_4$  (1.3 mM),  $\text{MgSO}_4$  (1.2 mM) and glucose (11.7 mM). The tracheal isolates were subjected to persistent stretch tensions through the attachment of 1 g of pre-loads and were maintained as such during experiments. The isolated tracheal fragment was incubated for 60 min for acclimatization before the addition of any test agent. The isometric tension of the isolates was measured through an isometric transducer (Model FORT 100, USA WPI) attached with a computerized

Power lab Data acquisition system (AD Instruments, Australia) using Lab Chart<sup>®</sup> software (v 8.0) (15). The tracheal isolates are the quiescent tissue preparations suitable for the screening of both agonists as well as antagonist activities. The tracheal isolate contracts on exposure to any muscarinic agonist (e.g. CCh) and can be used directly for the characterization of muscarinic agonists or antagonists. Alternatively, the relaxant effect of any test agent can be explored using CCh (1  $\mu$ M) or K<sup>+</sup> (80 mM) induced pre-contracted tracheal preparation. The test agents were added to the tissue organ baths in an additive manner seeking bronchodilator, muscarinic agonist, and muscarinic antagonist activities in comparison to the control. Concentration response curves (CRCs) for CCh were made on tracheal isolates in the presence of multiple concentrations of the test agents. The competitive antagonists on the muscarinic receptor exhibited the shifting of CRCs for CCh toward the right in a parallel manner maintaining heights of achievable responses (16, 17).

#### **Aortic isolates of rabbits**

The thoracic cavity of the sacrificed rabbit was incised to excise the thoracic aorta and was cut into 2-3 mm wide small pieces. It was then loaded promptly to the tissue organ bath containing 15 mL of Krebs's solution (37°C) and was bubbled consistently with carbogen gas. These aortic isolates were subjected to stretch tension on the application of a preload of 2 g and were then incubated for 60 min with the renewal of Krebs's solution at 10 min intervals for acclimatization. The responses of aortic isolates were measured through an isometric transducer (Model FORT100, USA WPI,) attached with a computer system installed with Lab Chart<sup>®</sup> software (v 8.0). The test agent can be applied to aortic isolates of rabbits in an additive manner for possible vasoconstrictor effect. However, the vasodilator effect can be determined on the application of test agents in an additive manner to P.E (1  $\mu$ M) or K<sup>+</sup> (80 mM) induced previous contractions (18).

#### **The paired atrial isolate of the rabbit**

The thoracic cavities of sacrificed rabbits were incised and the paired atria were isolated from the excised heart with extreme care. The paired atrial isolates were loaded to the tissue organ bath

containing 20 mL of Krebs's solution at 32°C and were bubbled continuously with carbogen gas. The atrial isolates demonstrated periodic rhythmic contractions on the application of stretch tension of 1 g because of the intact sinoatrial (SA) nodal cells. The tissue preparations were incubated for 30 min for acclimatization purposes with the replacement of old fluid by fresh Krebs's solution at 10 min intervals. The contractility of paired atrial isolates was measured through the Grass force-shift transducer (model FT-03) attached to computerized Power Lab (AD Instrument, Sydney, Australia) using Lab Chart<sup>®</sup> software. The contractile force of atria was estimated from the amplitudes of the recorded contractions; whereas, the rate of contractions of atria was determined through the frequency of contractions. The test agents were applied to paired atrial isolates to determine the possible influence on the force as well as the rate of contractions of atria (18).

#### **In vivo experiments**

##### **Anesthetized rats and measurements of blood pressure**

The normotensive adult Sprague-Dawley albino rats ( $\sigma/\text{♀}$ ) (230-300 g body weight; n= 5) were anesthetized by ketamine (i.p; 50-80 mg.kg<sup>-1</sup>) administration. These were used for the assessment of the possible antihypertensive effect of test agents (Cr.Cf) on blood pressure (BP) in accordance with the earlier reported method(19). The anesthetized animals were kept in a supine position. The hypothermia was avoided by means of an electric lamp. The trachea and right jugular vein were cannulated following insertion of 18-gauge, 50 mm polyethylene pipes following minor incisions at appropriate locations for the facilitation of respiration as well as intravenous administration of the test agents (Cr.Cf). The left carotid artery was cannulated by polyethylene tubes containing heparin solution (0.1 mL; 60 I.U.mL<sup>-1</sup>). It was connected to a pressure transducer (AD Instruments, Australia) coupled to a Power Lab using Lab Chart software<sup>®</sup> for the recording of blood pressure and heart rate. The animals were kept without medication (test agent or standard drug) for 20 min to achieve equilibration. The pressure in the left carotid artery was calibrated by a mercury sphygmomanometer attached to the transducer, each time before the start of experiments. Additionally, the

anti-coagulant effect was maintained with periodic injections of heparinized solution (0.1 mL). The control hypertensive and hypotensive responses were recorded on the intravenous injection of adrenaline ( $1 \mu\text{M.kg}^{-1}$ ) and acetylcholine ( $1 \mu\text{M.kg}^{-1}$ ) respectively. About 0.1 mL of the test agent was intravenously injected followed by flushing with 0.1 mL of normal saline. Moreover, returning the B.P to the resting level was mandatory before the administration of the next dose. At the end of the experiment, the animals were immediately euthanized via the cervical dislocation method to alleviate any pains.

### Acute toxicity studies in mice

Healthy albino mice ( $\sigma/\text{♀}$ ) weighing 20-30 g were obtained from the university animal house and were randomly divided into 4 groups, each group containing five animals [(n=5)  $\times$  4]. The animals were housed in a well-ventilated room with a 12 h light/dark cycle. The temperature was maintained at  $25 \pm 3^\circ\text{C}$  with free access to tap water. Prior to the oral administration of the Cr.Cf extract, all the animals were subjected to a short fasting period of 5 h. The extract was dissolved in normal saline and administered at a single dose of  $4 \text{ g.kg}^{-1}$ ,  $8 \text{ g.kg}^{-1}$ , and  $12 \text{ g.kg}^{-1}$ . The control group received normal saline only as a control. The general behaviour of the mice was continuously monitored for 6 h post-dosing, periodically during the first 24 h, and then daily thereafter for 14 days. Behavioural changes in mice were monitored on 1<sup>st</sup>, 3<sup>rd</sup>, 7<sup>th</sup>, and 14<sup>th</sup> days. Any signs of toxicity that appeared were recorded. All the protocols for acute and chronic toxicity studies were approved by the Ethical Research Committee, Bahauddin Zakariya University Multan, Pakistan (20).

### Chronic toxicity studies in rats

Sprague-Dawley albino rats weighing 150-210 g were randomly divided into four groups, each group containing five rats [(n=5)  $\times$  4]. The animals in the treatment group I, II, and III received Cr.Cf extract (dissolved in 0.9% NaCl) daily by gavage method at the respective dose of 250, 500, and  $1000 \text{ mg.kg}^{-1}$ . The control group (group IV) received NaCl (0.9%) only at a dose of  $10 \text{ mL.kg}^{-1}$  for 90 days. Any sign of toxicity and mortality was monitored daily during the 90 days of the experiment. After 90 days post-administration, the rats were anesthetized by

intraperitoneal injection of ketamine ( $50\text{-}80 \text{ mg.kg}^{-1}$ ). The blood from each rat was withdrawn into two sterile tubes via cardiac puncture. One tube contained EDTA (ethylenediamine-tetra acetic acid) as an anticoagulant while the other was without any anticoagulant. The blood without any additives was allowed to clot before centrifugation at 4000 rpm for 10 min at  $4^\circ\text{C}$  to obtain the serum. It was then analysed for serum biochemical indexes including alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, urea, creatinine and alkaline phosphatase (ALP) using commercially available kits (Redox laboratories, UK). The blood in the EDTA tubes was analysed immediately for hematological parameters including total erythrocytes count (RBCs), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), Total leukocyte count (WBCs) as well as the thrombocyte count (PLT) using haematology analyser (Icon-3, Norma Instruments, Budapest, Hungary). The rats were euthanized by cervical dislocation (immediately after blood sampling) for the gross pathological examinations of the heart, lungs, liver, kidney, and pancreas. The relative weight of each organ was calculated as:

$$\text{Body visceral index [\%]} = \frac{\text{weight of organ}}{\text{weight of rat}} \times 100 \quad (\text{i})$$

The histopathological examination of rats treated with  $1000 \text{ mg.kg}^{-1}$  of Cr.Cf was performed. The selected organs were fixed in 10% formalin. The organs were sliced into 4-5 mm thin tissue sections using a rotary microtome (Hunan Kaida scientific instruments, Changsha, China). These sections were fixed onto the glass slides and were stained with hematoxylin-eosin stain (H & E). These slides were observed microscopically (Olympus BX51M, Tokyo, Japan) for any sign of pathological changes in the organs using established protocols (21).

### Statistical Analysis

All the results are presented as mean  $\pm$  SEM (standard error mean). The  $\text{EC}_{50}$  values (median estimated response) with a 95% confidence were determined with the Graph Pad Prism<sup>®</sup> 6 (San Diego,

USA). Moreover, the concentration-response curves (CRCs) were constructed using a non-linear regression sigmoidal plot. For the *in vivo* experiments, One-way ANOVA (Analysis of variance) with post-hoc Dunnett's test (single comparison with control) was applied.

## Results and discussion

### Phytochemical detection of crude pod extract (*Cr.Cf*)

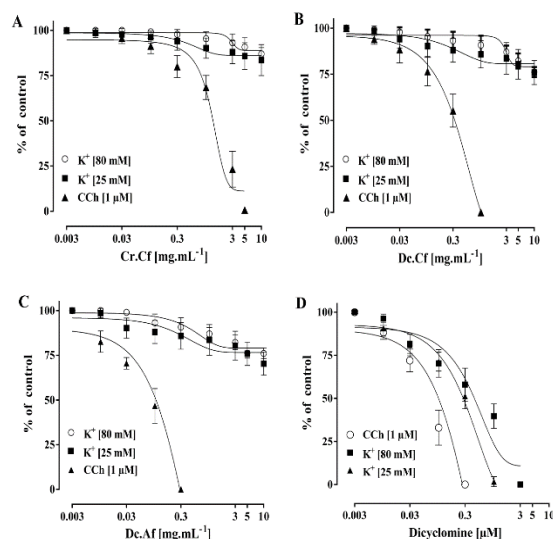
Preliminary analysis demonstrated the presence of alkaloids, flavonoids, phenols, saponins, and tannins in the crude extract (*Cr.Cf*). The alkaloids, resins, fats, and waxes were also detected in the dichloromethane fraction (*Dc.Cf*) of the extract.

### Ex- vivo experiments

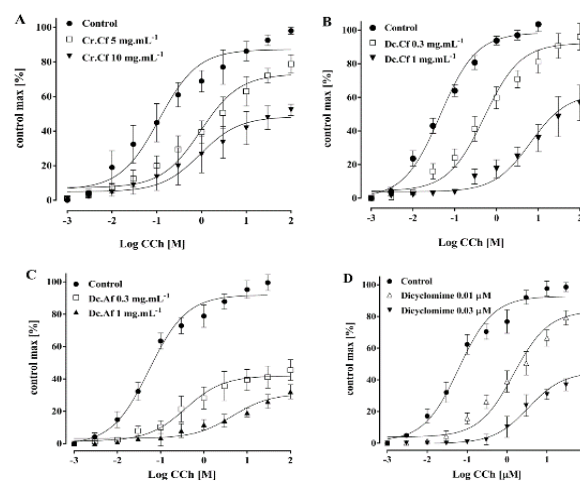
#### Tracheo-relaxant activity on the tracheal fragment of rabbits

The hydro-methanol extract of *C. siliqua* (*Cr.Cf*), dichloromethane fraction (*Dc.Cf*) and alkaloid fraction (*Dc.Af*) relaxed the carbachol (CCh; 1 μM)-induced contractions in the tracheal fragment with respective EC<sub>50</sub> of 4.78 mg.mL<sup>-1</sup> (95% CI; 3.03-7.56 mg.mL<sup>-1</sup>), 1.083 mg.mL<sup>-1</sup> (95% C.I; 0.62-1.87 mg.mL<sup>-1</sup>) and 0.19 mg.mL<sup>-1</sup> (95% CI; 0.11- 0.34 mg.mL<sup>-1</sup>). The extract and fractions were unable to relax the stimulated contractions caused by K<sup>+</sup> (80 mM) and K<sup>+</sup> (25 mM) in the tracheal fragment of rabbits. Dicyclomine (standard) demonstrated the broncho-relaxant activity on CCh (1 μM), K<sup>+</sup> (80 mM) and K<sup>+</sup> (25 mM)-stimulated contractions in the tracheal fragments of rabbits with respective EC<sub>50</sub> values of 0.095 μM (95% C.I; 0.06–0.14 μM), 0.75 μM (95% C.I; 0.38-1.49 μM) and 0.26 μM (95% C.I; 0.02-2.49 μM) (Fig 1).

Furthermore, the crude extract (*Cr.Cf*; 5-10.0 mg.mL<sup>-1</sup>), dichloromethane fraction (*Dc.Cf*; 0.3-1.0 mg.mL<sup>-1</sup>) and alkaloid fraction (*Dc.Af*; 0.3-1.0 mg.mL<sup>-1</sup>) demonstrated the parallel shifting of CRCs of CCh towards right with decrease of the maximum contractile effect in a fashion similar to dicyclomine (0.01-0.03 μM) on tracheal fragments of the rabbits (Fig 2).



**Figure 1.** Dose-response nonlinear curves representing the effect of *Ceratonia siliqua* on the tracheal fragment of rabbits (A) crude extract (*Cr.Cf*); (B) dichloromethane fraction (*Dc.Cf*); (C) alkaloids base concentrate (*Dc.Af*) and (D) the Dicyclomine on the contractions exerted by the Carbachol [CCh -1 μM], Potassium [K<sup>+</sup>- 80 mM] and [K<sup>+</sup>- 25 mM] in the tracheal fragment of rabbits. The values are presented as mean ± SEM (n=5).

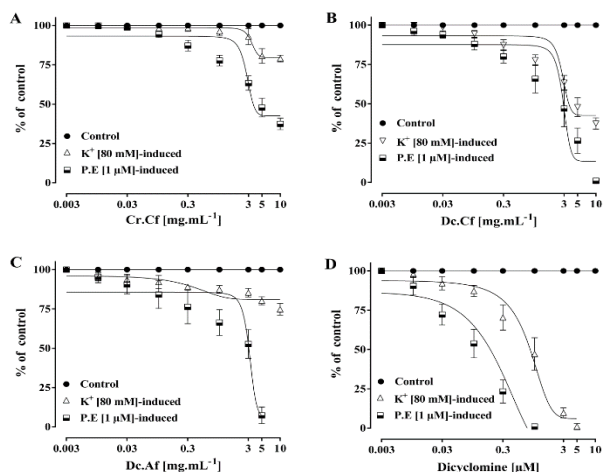


**Figure 2.** Graphic representation of the effect of *Ceratonia siliqua* on tracheal isolates of rabbits (A) The crude extract; (B) Dichloromethane fraction; (C) Alkaloids base concentrate and (D) The Dicyclomine on concentration-response curves (CRCs) of Carbachol [CCh] in the tracheal isolates of rabbits. The values are expressed as mean ± SEM (n=5).

#### Vaso-relaxant activity on the aortic fragment of rabbits

The test substance *Cr.Cf* completely relaxed the P.E. (1 μM)-stimulated contraction, and partially relaxed the K<sup>+</sup> (80 mM)-mediated contraction in the

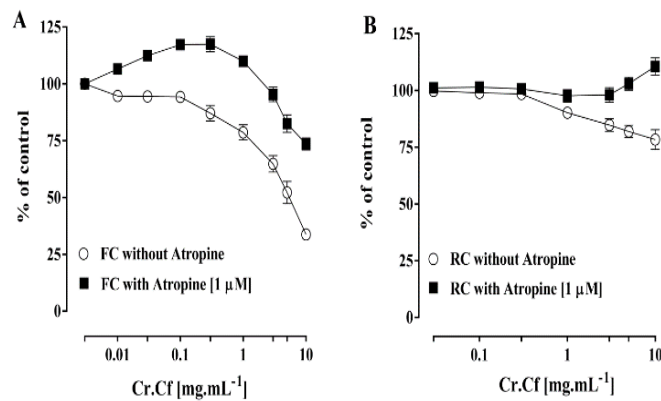
aortic fragment of the rabbits with the respective  $EC_{50}$  values of  $3.03 \text{ mg.mL}^{-1}$  (95% CI;  $2.05\text{-}4.47 \text{ mg.mL}^{-1}$ ) and  $9.63 \text{ mg.mL}^{-1}$  (95% CI;  $3.17\text{-}19.2 \text{ mg.mL}^{-1}$ ). The Dc.Cf also followed the crude extract pattern and completely relaxed the P.E. ( $1 \mu\text{M}$ )-stimulated contraction as also partially relaxed the  $K^+$  ( $80 \text{ mM}$ )-mediated contraction with the respective  $EC_{50}$  values of  $4.74 \text{ mg.mL}^{-1}$  (95% CI; of  $2.85\text{-}7.88 \text{ mg.mL}^{-1}$ ) and  $3.10 \text{ mg.mL}^{-1}$  (95% CI;  $2.25\text{-}4.49 \text{ mg.mL}^{-1}$ ). The Dc.Af fraction failed to induce relaxant effect in the P.E. ( $1 \mu\text{M}$ )-stimulated contraction. But it showed partial relaxation of  $K^+$  ( $80 \text{ mM}$ )-mediated contraction with  $EC_{50}$  value of  $2.91 \text{ mg.mL}^{-1}$  (95% CI;  $10.02\text{-}8.26 \text{ mg.mL}^{-1}$ ). Dicyclomine induced the vasorelaxant effect by relaxing the P.E ( $1 \mu\text{M}$ ) and  $K^+$  ( $80 \text{ mM}$ )-induced contractions in the aortic fragment of rabbit with the  $EC_{50}$  values of  $1.23 \text{ mg.mL}^{-1}$  (95% CI;  $0.91\text{-}1.66 \text{ mg.mL}^{-1}$ ) and  $0.12 \text{ mg.mL}^{-1}$  (95% CI;  $0.087\text{-}0.188 \text{ mg.mL}^{-1}$ ) respectively (Fig 3).



**Figure 3.** Graphical representation of the effect of *Ceratonia siliqua* on aortic isolates of rabbits (A) The crude extract; (B) Dichloromethane fraction; (C) Alkaloids base concentrate; (D) The Dicyclomine on the contractions caused by phenylephrine [P.E -  $1 \mu\text{M}$ ] and potassium [ $K^+$ -  $80 \text{ mM}$ ] in the aortic isolates of rabbits. The values are presented as Mean  $\pm$  SEM (n=5).

### Cardiac activity on paired atria of rabbits

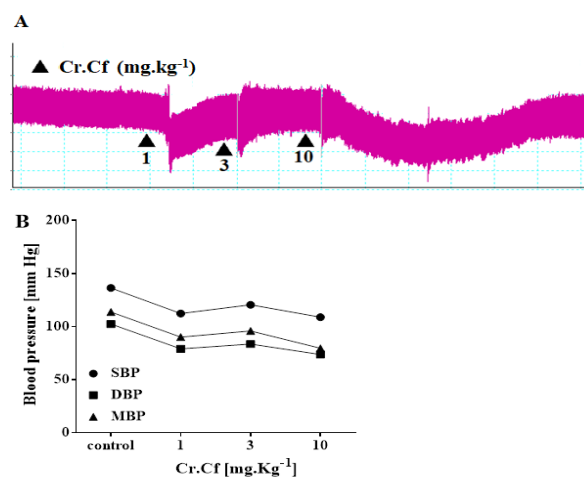
The Cr.Cf induced negative inotropic and chronotropic effects in the spontaneously contracting paired atrial fragments of rabbits at the tissue bath concentration of  $10 \text{ mg.mL}^{-1}$ . These negative inotropic and chronotropic effects induced by Cr.Cf became blunt upon pre-treatment with atropine ( $1 \mu\text{M}$ ) (Fig 4).



**Figure 4.** The effect of *Ceratonia siliqua* crude extract on isolated rabbit atria (A) The force of contraction (FC), and (B) the rate of contraction (RC) in the absence and the presence of atropine [ $1 \mu\text{M}$ ] on the paired atrial isolates of the rabbits. The values are presented as mean  $\pm$  SEM (n=5).

### Hypotensive activity in normotensive anesthetized rats

The Cr.Cf fraction of the extract induced hypotensive response in terms of the reduction in systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MBP) on intravenous administration to normotensive anesthetized rats (Fig 5).



**Figure5.** Pictorial illustration of *Ceratonia siliqua* on blood pressure regulation in normotensive rats (A) Tracing showing the effect of crude extract [ $1\text{-}10 \text{ mg.kg}^{-1}$ ] on the blood pressure after intravenous administration and (B) the effect of extract on systolic blood pressure [SBP], diastolic blood pressure [DBP] and mean arterial blood pressure [MBP] of the normotensive anesthetized rats. The values are presented are mean  $\pm$  SEM (n=5).

**Table 1.** Effect of different doses of hydro-methanol extract of *Ceratonia siliqua* pods on the biochemical parameters of the rats, Biochemical Parameters (A), Reference Range (B), Control (C), 250 mg.kg<sup>-1</sup> extract (D), 500 mg.kg<sup>-1</sup> extract (E), 1000 mg.kg<sup>-1</sup> extract (F)

A	B	C	D	E	F
Urea (mg/dL)	10.71 - 20.00	18.37± 0.50	19.00± 0.44 <sup>d</sup>	19.20± 0.37 <sup>d</sup>	19.80± 0.20 <sup>d</sup>
Creatinine (mg/dL)	0.30 - 0.50	0.38 ± 0.03	0.42± 0.02 <sup>d</sup>	0.42± 0.03 <sup>d</sup>	0.48± 0.02 <sup>d</sup>
Bilirubin (mg/dL)	0.04 - 0.20	0.06 ± 0.01	0.09± 0.01 <sup>d</sup>	0.12± 0.01 <sup>a</sup>	0.14± 0.02 <sup>b</sup>
SGPT/ALT (U/L)	19.00- 48.00	38.80± 1.59	37.60± 0.92 <sup>d</sup>	42.20± 1.10 <sup>d</sup>	42.50± 1.63 <sup>d</sup>
SGOT/AST (U/L)	63.00- 175.00	132.00± 2.90	135.00± 2.70 <sup>d</sup>	148.00± 1.90 <sup>b</sup>	150.00± 1.70 <sup>c</sup>
ALP (U/L)	36.00- 131.00	104.20± 3.20	111.00± 2.70 <sup>d</sup>	115.00± 2.72 <sup>d</sup>	121.60± 4.65 <sup>b</sup>

The Values are expressed as mean ± SD (n = 5). Treatment groups were compared to the normal saline (NC). The significance levels are expressed as: a (p < 0.05), b (p < 0.01), c (p < 0.001) and d (non-significant).

## In vivo experiments

### Acute oral toxicity studies

After the administration of Cr.Cf, all the mice were monitored for a short duration (24 h) as well as for a relatively long duration (14 days). The dose of 4 g.kg<sup>-1</sup> and 8 g.kg<sup>-1</sup> did not show any clinical sign of acute toxicity in the test subjects for shorter or longer durations). The mice at a dose of 12 g.kg<sup>-1</sup> however showed the signs of toxicity including increased defecation, decreased mobility, piloerection, as well as the stretching of forelimbs and were abolished at the end of the experiment. No mortality was recorded until the end of the study.

### Chronic toxicity studies

#### Relative organ weight ratio (Body visceral index)

The organ body weight ratio of rat's heart, lungs, liver, right kidney, left kidney, and pancreas in the treatment groups was compared to the control group. No significant change in the body visceral index was observed for the heart, liver, pancreas, and kidney. However, a slight increase in the size of the lungs was found at a high investigated dose (i.e. 1000 mg.Kg<sup>-1</sup>).

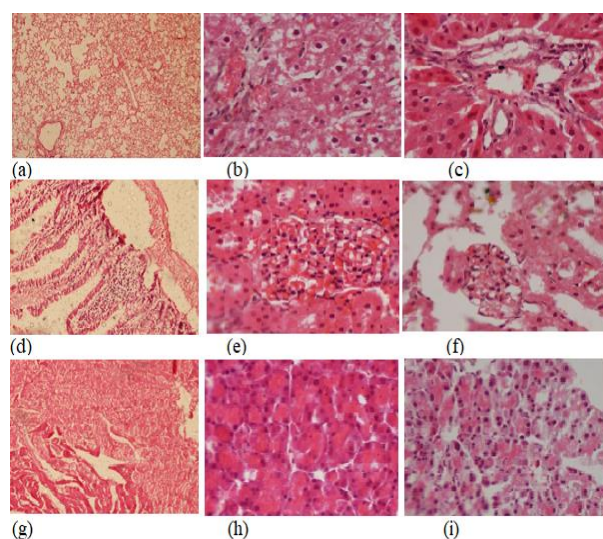
### Haematological parameters

The effects of different Cr.Cf doses on the haematological parameters were tabulated. All the

haematological parameters were found to be within the reference range. An insignificant difference was found in the case of WBC, MCV, and MCH values. However, a significant difference in the levels of RBC, HCT, MCHC, and platelets was found at a dose of 250 and 500 and 1000 mg.kg<sup>-1</sup> in comparison to the control group.

### Biochemical parameters

Treatment of the test subjects with different Cr.Cf doses did not show any significant difference in the hepatic and renal biomarkers including ALT, AST, ALP, urea, creatinine as well as bilirubin levels as compared with reference values. However, in comparison to the control group, the bilirubin, AST and ALP levels were significantly (p; <sup>a</sup> < 0.05, <sup>b</sup> < 0.01) increased at a dose of 500 and 1000 mg.kg<sup>-1</sup> (Table 1).



**Figure 6.** Histopathology of tissue sections from rats chronically exposed to 1000 mg/kg/day *Ceratonia siliqua* extract at 40X magnification (a) Lungs (b) Liver (c) Damaged liver (d) Intestine (e) normal kidney (f) damaged kidney (g) heart (g) heart (h) normal pancreas (i) damaged pancreas of rats

### Histopathological examination

The histopathological examination of the heart, intestine and lungs showed normal histology in animals treated with 1000 mg.kg<sup>-1</sup> dose of the plant extract. However, animals treated with 1000 mg.kg<sup>-1</sup> of Cr.Cf showed necrosis of hepatocytes, damage to the glomerular and tubular structure of nephrons,



slight necrosis of Acinar cells and degeneration of pancreatic lob (Fig 6).

### Conclusion

In conclusion, the crude extract of *C. siliqua pods* (Cr.Cf) demonstrated the bronchodilator potential possibly mediated through muscarinic receptors antagonism. Moreover, vasodilator and hypotensive effects are speculated to be mediated through  $\alpha$  adrenoceptor antagonism and involvement of calcium channel blocking as well as cardio-selective muscarinic agonistic activities. These investigations provided the scientific basis for the validation of the folkloric use of *C. Siliqua pods* in the management of ailments regarding respiratory and cardiovascular disorders. Furthermore, *C. siliqua pods* (Cr.Cf) did not cause any sign of toxicity in acute and chronic oral toxicity examinations.

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### Interest conflict

The authors declare no conflict of interest.

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