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Research Article

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Some Pharmacological Tests of *Datisca cannabina* Linn

Zainab[a,b], Abdul Aziz[c], Muddaser Shah[d,e], Noor Muhammad[b,f], Mehjabeen[g], Noor Jahan[h], Najeeb Ur Rehman[d*], Ahmed Al-Harrasi[d*], Manzoor Ahmad[b*]

[a] *College of Chemistry and Materials Science, Hebei Normal University, Shijiazhuang 050024, China.*
[b] *Department of Chemistry, University of Malakand, Chakdara, Dir (L), 18800, Pakistan.*
[c] *Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, Peoples Republic of China.*
[d] *Natural & Medical Sciences Research Center, University of Nizwa, Nizwa 616, Oman.*
[e] *Department of Botany, Bacha Khan University, Charsadda 24420, Khyber Pakhtunkhwa, Pakistan.*
[f] *Drug Regulatory Authority, Islamabad, Pakistan.*
[g] *Department of Pharmacology, Federal Urdu University of Arts, Science & Technology, Karachi-75300, Pakistan.*
[h] *Department of Pharmacology, College of Pharmacy, Dow University of Health Sciences Karachi, Pakistan.*

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Abstract

In the current study, methanolic extracts (DCME) along with their fractions (n-hexane (DCHF), chloroform (DCBF), ethyl acetate (DCEF), n-butanol (DCBF), and aqueous (DCAF) of *Datisca Cannabina* Linn. were examined for in vitro biological activities, i.e., phytotoxicity, cytotoxicity, antioxidant, hemagglutination bioassay, urease, and spasmogenic and spasmolytic activities. Our findings revealed that the study plant is non-toxic, as neither its crude extract nor its subfractions exhibited toxic effects against brine shrimp. Additionally, no significant insecticidal activity was observed in the crude extracts or subfractions. Furthermore, the crude extract showed significant analgesic potential compared to the other tested samples, indicating the presence of bioactive constituents in the crude methanol extract. In the case of hemagglutination activity, all extracts exhibited a high (+++) or medium (++) agglutination effect against all human blood groups at a 5 mg/mL concentration, except for blood groups B and A, where the effect was low. Similarly, the crude methanol and aqueous fractions were found to have maximum (90%) and minimum (80%) phytotoxic activity, respectively, at a higher concentration (1000 µg/mL) only. In the case of urease enzyme inhibition studies, the ethyl acetate fraction showed maximum inhibition (90%), followed by DCBF (88%) and DCAF (70%), while the other fractions were found to be inactive. Among all the fractions screened, the same fraction (DCEF, 86.0%) displayed the highest antioxidant activity, followed by the chloroform fraction (75%) compared to standard BHA (92.25%) at a concentration of 1.0 mg/ml. In terms of spasmogenic and spasmolytic significance, *D. cannabina*'s fractions were found to have a mild contracting and relaxing effect, varying from low to high doses, which may explain the traditional use of the plant in treating constipation and abdominal cramps, respectively.

Keywords: *Datisca cannabina* Linn.; Analgesic potential; Phytotoxicity; Cytotoxicity; Antioxidant; Urease; Smooth muscle activities.

*** Corresponding Authors**:
Najeeb Ur Rehman, Natural & Medical Sciences Research Center, University of Nizwa, Nizwa 616, Oman. E-mail: najeeb@unizwa.edu.om
Ahmed Al-Harrasi, Natural & Medical Sciences Research Center, University of Nizwa, Nizwa 616, Oman. E-mail: aharrasi@unizwa.edu.om
Manzoor Ahmad, Department of Chemistry, University of Malakand, Chakdara, Dir (L), 18800, Pakistan.. E-mail: manzoorahmad@uom.edu.pk.

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1. Introduction

Medicinal plants and their resins have a wide range of traditional and modern therapeutic applications, making them highly valuable in the healthcare industry. Awareness of medicinal plant utilization is the consequence of many years of struggle against diseases, and man learnt to seek medications in barks, seeds, fruits, and other parts of plants [1]. These plants offer substantial economic and health benefits, particularly in developing nations with high poverty rates and limited healthcare coverage [2]. According to the World Health Organization (WHO), traditional medicine is utilized by 80% of the global population, and there has been a consistent demand for and widespread use of traditional and complementary medicine worldwide [3]. Plants produce a wide array of chemical compounds that serve various functions, such as defense against insects, fungi, bacteria, diseases, and herbivorous mammals. Plants are a rich natural supply of chemical constituents that could provide the basis for the creation of new medicines. Phytomedicines, collectively comprising plants with medicinal properties and their chemical constituents, play a significant therapeutic role in addressing various health-related issues, including gastrointestinal infections, free radical scavenging, and antidiabetic properties [4]. Recognizing the importance of medicinal plants, numerous research groups are actively involved in medicinal plant research.

Natural antioxidants are extensively distributed in foods and medicinal plants. These natural antioxidants have a variety of medicinal impacts, including anti-inflammatory, anti-atherosclerotic, anti-aging, and anticancer properties [5]. Numerous studies have emphasized that various natural plant-based antioxidant molecules have been useful in resolving health issues associated with oxidative stress. Some plant-based antioxidants have exhibited promising biological effects, particularly in combating human pathogenic microorganisms, minimizing pain, and alleviating inflammation [6, 7]. Recently, considerable research has focused on the utilization of natural antioxidants to protect the human body against brain tissue damage and neurological problems associated with free radical damage.

Urease enzymes play a crucial role in catalyzing the hydrolysis of urea, garnering significant attention regarding human health and their impact on quality of life [4]. *Helicobacter pylori* infection is the leading cause of gastrointestinal problems, including gastritis, peptic ulcers, duodenal ulcers, and gastric ulcers. The enzyme neutralizes gastric acid by hydrolyzing urea, producing CO_2 and NH_3 [8]. Marketed urease medicines are substantially more toxic, with lower efficacy rates, which restricts their clinical application [9, 10]. Therefore, the search for novel urease inhibitors with enhanced stability and reduced toxicity is necessary to improve the quality of life for humans and animals. This study aims to identify new sources of natural antioxidants and urease inhibitors that can serve as lead chemical substances in the discovery of new medications, either alone or in combination with existing approved treatments.

Pain is a major public health concern that affects 20% of adults worldwide, with 1 in every 10 adults experiencing chronic pain annually [11]. Pain can be caused by a variety of medical disorders, such as cancer, osteoarthritis, rheumatoid arthritis, surgeries, spinal difficulties, and traumas. Understanding the pathophysiology of pain can be challenging [12]. It is an unpleasant sensation associated with actual or potential tissue damage that activates certain nerve fibers and delivers a signal to the brain, where its awareness may be altered by various circumstances [13]. Current treatments for inflammation and pain are helpful, but they have serious adverse effects such as ulcers, osteoporosis, anemia, and endocrine disruption [14]. Since ancient times, medicinal herbs have been employed as analgesics [15]. As synthetic medications are expensive and have some adverse side effects, plants are being examined as alternatives for screening analgesic drugs [16]. Research on plants with medicinal properties, including analgesic activities, is necessary and can serve as a basis for treating various disorders and complications [17].

Datisca cannabina Linn., (Datiscaceae) is a glabrous herb by habit, mostly preferred to grow in light with loamy, acidic, neutral, basic, and well-drained soils [18]. The genus *Datisca* is cosmopolitan in its habitat, distributed across the tropical and subtropical western Himalayas, including Kashmir, Nepal, Turkey, Afghanistan, and Pakistan [19]. Locally practiced as an effective remedy to cure fevers, gastric, and scrofulous complications [20]. It also possesses healing properties, including the ability to relieve joint disorders, act as an expectorant, and alleviate toothache [18]. Pakistan has a

rich biodiversity, with plants attributed to multiple health benefits, ranging from local practices to their diverse commercial applications [6]. The attention toward herbal therapies has increased as they are effective, economical, and have fewer side effects [21]. Many of them have been explored for their pharmacological activities, including antimicrobial, antioxidant, antidiabetic [22], analgesic, anti-inflammatory, and anticancerous potential [22]. However, we still need to investigate the unexplored plants and highlight their significance due to their valuable medicinal purposes [23, 24]. In this context, as part of our ongoing studies on exploring the hidden potential of Pakistan's indigenous flora [25]. Hence, *D. cannabina* various extracts were profiled for their phytomedicinal potential.

Given the traditional relevance of the understudy plant, this study is being conducted for the first time to investigate it's in vitro and in vivo properties, including antioxidants, cytotoxic, phytotoxic, urease, insecticidal, and analgesic potentials. The present investigation will provide a broad foundation for further detailed pharmacological and phytochemical studies on D. cannabina, along with its biological standardization. The active compounds in this plant may be candidates for treating a variety of maladies, including ulcers, Alzheimer's disease, cytotoxicity, and oxidative stress-related issues.

2. Material and Methods

2.1. Chemicals and Reagents

Diazepam (5 mg tablets), Diclofenac sodium, paraquat, carrageenan, acetone, acetic acid, saline, distilled water, and a plethysmometer (7150 Ugo Basile) were used. Acetylcholine perchlorate, adrenaline, and atropine were purchased from Sigma Chemicals Co., St. Louis, MO, USA. All chemicals used were of the highest purity grade. Stock solutions of all the chemicals were made in distilled water, and the dilutions were made fresh on the day of the experiment. The vehicle used for the solubilization of drugs did not affect tissue contractility in the control experiments.

2.2. Collection and Identification of Plant

Various field visits were conducted from August to September 2021 to collect the entire plant of *D. cannabina* from Matta, Swat District, Khyber Pakhtunkhwa, Pakistan. They were identified by Prof. Mehboob Ur Rehman (Taxonomist), Jahan Zeb Post Graduate College, Saidu Sharif, Swat, Pakistan. A voucher specimen (CD-01/2021) was deposited in the herbarium of the Department of Botany, Jehan Zeb Postgraduate College, Saidu Sharif, Swat, Pakistan, and in the herbarium of the University of Karachi.

2.3. Extraction and Fractionation

The collected plant samples (6.0 Kg) were washed to remove impurities and placed in the shade to dry. The air-dried aerial parts (4.5 kg) of *D. cannabina* were soaked in methanol (7.5 L) at room temperature for 15 days (3 times). The process was repeated twice, and the three residues obtained after filtration of the percolates were combined. Methanol was evaporated under reduced pressure at 40 ℃, and the crude extract obtained was lyophilized, yielding 260 g of extract. The crude extract (DCMF) (30 g) was kept for biological and pharmacological screening, whereas the 230 g was subjected to further fractionation. The crude methanolic extract was homogenized entirely with distilled water (1 L). It was then further fractionated into *n*-hexane (DCHF, 35 g), chloroform (DCCF, 55 g), ethyl acetate (DCEF, 28 g), *n*-butanol (DCBF, 46 g), and aqueous (DCAF, 60 g) fractions. All the crude extract and subfractions were appropriately capped and placed in the refrigerator till further use [26].

2.3.1. Hemagglutination Activity

The blood was obtained from Husaini Blood Bank, Karachi, and the erythrocytes were used [27]. The hemagglutination activity was investigated at various concentrations of the crude extract against human erythrocytes of blood groups A, B, AB, and O (Rh+ and Rh-) [28]. Weak, moderate, and strong agglutinations were determined based on the extent of granular deposition.

2.3.2. Brine Shrimp Lethality Bioassay

It is an excellent and straightforward preliminary method for determining the cytotoxicity of crude plant extracts and natural compounds [29]. In this method, artificial

"sea water" was prepared by dissolving 3.8 g of sea salt per liter of double-distilled water and filtering [30]. "Seawater" was placed in a small tank; brine shrimp eggs (1mg) (*Artemia salina*) were darkened by covering them with aluminum foil. It was allowed to stand for 24 hours at 25 °C, which resulted in many larvae. Twenty milligrams of the concentrated sample were dissolved in 2 mL of CHCl3 (20 mg/2 mL) and transferred to 5, 50, and 500 µL vials, corresponding to concentrations of 10, 100, and 1000 µg/mL, respectively. Then, three replicates were prepared for each concentration, resulting in a total of nine vials. The vials containing the material were concentrated and then dissolved in DMSO (50 µL). Five milliliters of "seawater" were added to each. Then 10 shrimps were added per vial, allowed to stand for 24 hours, and the number of surviving shrimps was counted. Etoposide was used as a positive control. The data were analyzed with a Finney computer program to determine the LD_{50} values.

2.4. *Phytotoxic activity*

The phytotoxic capabilities of the under-study plant were evaluated using Lemna minor with slight modifications [31, 32]. It is a floating aquatic plant that is used to test the inhibitory or stimulating effects of bioactive components in crude extracts. The medium was prepared by mixing various constituents in 100 mL of distilled water, and the pH was adjusted (5.5-6.5) by adding KOH solution. The medium was then autoclaved at 121℃ for 15 minutes. The extracts were dissolved in methanol (20 mg/mL) and served as the stock solution. Nine sterilized flasks, three for each concentration, were inoculated with 1000 µL, 100 µL, and 10 µL of the stock solution for concentrations of 500, 50, and 5 ppm, respectively. The solvent was allowed to evaporate overnight under sterile conditions. To each flask, medium (20 ml) and plants (10), each containing a rosette of three fronds of *Lemna minor* L., were added. All flasks were plugged with cotton and kept in the growth cabinet for 7 days. The result was calculated in relation to the positive and negative controls. Paraquat was utilized as the standard medication, with volatile solvents operating as positive and negative controls [32]. The number of fronds per flask was counted and recorded on day seven, and the following formula was used to calculate their growth regulation in percentage:

$$\% \text{ Growth Regulation} = \frac{100 - \text{No.of fronds in test}}{\text{No.of fronds in –ve control}} \times 100$$

2.5. *Insecticidal activity*

The tested sample, crude extract, and subfraction extracts of D. cannabina, were evaluated against different insects, viz., *Tribolium castaneum*, *Callosbruchus analis*, and *Rhyzopertha dominica*. The samples were prepared by dissolving 200 mg of crude extract in 3 mL of acetone and loading them into Petri dishes covered with filter papers. After 24 hours, 10 test insects were placed in each plate and incubated at 27 ℃ for 24 hours with 50% relative humidity in the growth chamber. The results were analyzed as percentage mortality, calculated in relation to the positive and negative controls. Permethrin was used as a standard drug, while permethrin, acetone, and test insects were used as positive and negative controls [32]. The formula calculated the percentage mortality:

$$\% \text{ Growth Regulation} = \frac{100 - \text{No.of insects alive in test}}{\text{No.of insects alive in control}} \times 100$$

2.6. *Antioxidant Activity*

The antioxidant potential of *D. cannabina* extracts was examined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical bioassay [33]. The reaction mixture, containing five µL of the test sample (200 µg/mL in DMSO) and 95 µL of DPPH (Sigma, 300 µM in ethanol), was then placed in a 96-well microtiter plate (Molecular Devices, USA) and incubated in an ELISA at 37 °C for 30 min. The absorbance was measured at 515 nm. Percent radical scavenging activity was determined by comparison with a DMSO-treated control.

$$\text{DPPH scavenging activity (\%)} = A_{\text{control}} - A_{\text{test sample}}/Ao \times 100$$

where Ao is the absorbance of the control sample and Ai is the absorbance of the test sample.

2.7. *Urease bioassay*

The urease assay was performed using a reported method, as documented by Akhtar et al. [34], with slight modifications. A solution comprising 25 µL of Jack bean Urease, 55 µL of buffer, and 100 mM urea was incubated

with 5 µL (0.5 mM concentration) of the test compounds at 30 °C for 15 min in well plates. The production of ammonia was measured using the indophenol method to determine urease inhibitory activity. The phenol reagent (45 µL, 1% w/v phenol and 0.005% w/v sodium nitroprusside) and alkali reagent (70 µL, 0.5% w/v sodium hydroxide and 0.1% NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). The change in absorbance per minute was noted, and the results were processed using SoftMax Pro software (Molecular Devices, USA). All the tests were performed in triplicate. The assays were performed at pH 8.2 (0.01 M $K_2HPO_4.3H_2O$, 1.0 mM EDTA, and 0.01 M $LiCl_2$). Thiourea was used as the standard inhibitor of urease [34, 35]. The % inhibition was calculated from the formula.

$$\text{Inhibition (\%)}\ 100 - (OD_{\text{test sample}}/OD_{\text{control}}) \times 100$$

2.8. Spasmogenic and spasmolytic activity test

2.8.1. Smooth muscle preparation

Rabbits (1.0–1.5 kg) of either sex and local breed were housed at the Animal House of the University of Karachi, maintained at 23–25 °C, and were given a standard diet and tap water. Experiments performed complied with the rulings of the Institute of Laboratory Animal Resources, Commission on Life Sciences, and National Research Council (NRC, 1996). Animals had free access to water, but the food was withdrawn 24 h before the experiment. Dose concentrations of 1, 5, 10, 15, 20, and 25 mg/mL were used in the experiment. A blow on the back of the neck sacrificed the rabbit. The abdomen was opened immediately, and the caecum was pulled forward to display the length of the small intestine. The intestine was then cut from the animal and placed in a Petri dish or beaker containing Tyrode's solution [36, 37].

2.8.2. Isolated intestine segments preparation

The segments of the small intestine (jejunum or ileum), approximately 3-4 cm long, were separated immediately from the isolated intestine. They were then placed in a petri dish or beaker containing Tyrode's solution. For experimentation, a piece of isolated smooth muscle was mounted in an organ bath of 70 mL capacity, filled with Tyrode's solution. The organ bath circulating water temperature was maintained at 37°C throughout the experiment. The perfusion solution was bubbled with a mixture of 95% oxygen and 5% carbon dioxide. The intestine segment was allowed to equilibrate before starting the experiments. The spontaneous movements of the intestine were recorded on an oscillograph or polygraph using an isotonic transducer [36]**.** To determine the effects of plant extract on spontaneous movements of the intestine, crude extract and its fractions were dissolved in 1 mL of distilled water, and thereafter, it was added to the organ bath after the equilibration period. The effects of crude extract and its fractions on the contraction and relaxation pattern of isolated rabbit intestines (smooth muscles) were recorded.

2.9. Analgesic screening (Hot plate method)

The test was performed according to the modified method of Koster et al. [38]. According to this method, writing was induced by intraperitoneal administration of an acetic acid solution at a dose of 10 ml/kg. Thirty minutes prior to the administration of acetic acid, the mice were treated orally with the test substance. The number of writhes was counted for 30 minutes immediately after acetic acid administration. A reduction in the number of writhes compared to the control animals was considered evidence for the presence of analgesia and expressed as a percentage inhibition of writhing. Mice were divided into four groups (i.e., Group A for the control, Groups B and C for 300 mg/kg and 500 mg/kg oral doses of the crude extract, respectively, and Group D). Each group comprised five animals, weighing 25-34 g. Diclofenac sodium, administered orally at 50 mg/kg, was used as the standard. The crude drug and the diclofenac sodium were diluted in distilled water and administered orally. The control mice were treated orally with the same volume of saline as the crude extract. The study was completed in accordance with the guidance provided for strict adherence to the protocol recommended by the Malakand University Ethics Committee, with reference number (Pharm/EC/22/012, Dated: 10.10.2022).

3. Results and Discussion

Statistical Analysis

The results were expressed as mean ± SEM. All statistical comparisons were made using Student's *t*-test, and a P-value less than 0.05 was considered significant.

3.1. *Hemagglutination activity*

The hemagglutination potential of the *D. cannabina* crude extract was tested against various groups of human erythrocytes (**Table 1**). The extract exhibited a high (+++) or medium (++) agglutination effect against all human blood groups at a concentration of 5 mg/mL, except for blood groups B and A, where the effect was low. However, no agglutination properties were exhibited by this extract at concentrations of 1.25, 0.625, and 0.3125 mg/mL. It is well known that the hemagglutination activity is mainly related to a group of proteins called lectins, as stated by Benevides et al. [39], which are valuable tools for the separation and characterization of glycoconjugates and glycopeptides, histochemistry of cells and tissues, and the study of cell differentiation, as reflected in the literature of Gabius et al. [40]. This preliminary investigation revealed that *D. cannabina*, possessing hemagglutination activity, may contain valuable phytolectins that could find applications in the aforementioned areas.

Table 1. *In vitro* hemagglutination activity of the *D. canabinna*

Blood group	Dose of Drug (mg/mL)				
	5	2.5	1.25	0.625	0.3125
A^+	++	++	-	-	-
B^+	++	++	-	-	-
O^+	++	++	-	-	-
AB^+	++	-	-	-	-
A^-	+	-	-	-	-
B^-	+	-	-	-	-
O^-	+++	++	-	-	-
AB^-	++	+	-	-	-

Key: high (+++) or medium (++) agglutination effect against all human blood groups at 5 mg/mL concentration except blood group B^- and A^-, where it is low.

3.2. *Brine shrimp lethality bioassay*

LD_{50} measurements of crude as well as its fractions were evaluated against *Artemia salina* brine-shrimp eggs. It was evident from the results that neither crude extract nor any fraction had any activity as compared to standard drugs (**Table 2**).

Table 2. *In vitro* cytotoxic activity of *D. canabinna*.

Tested samples	Conc.	No. of survivors	Control	Results
DCHF	1000	30	30	-
DCCF		29		-
DCEF		27		+
DCBF		29		-
DCAF		30		-
DCMF		30		-
DCHF	100	30	30	-
DCCF		30		-
DCEF		29		-
DCBF		30		-
DCAF		30		-
DCMF		30		-
DCHF	10	30	30	-
DCCF		30		-
DCEF		30		-
DCBF		30		-
DCAF		30		-
DCMF		30		-

Crude methanol extract (DCMF), n-hexane fraction (DCHF), chloroform fraction (DCCF), ethyl acetate fraction (DCEF), n-butanol fraction (DCBF), and aqueous fraction (DCAF).

3.3. *Phytotoxic bioassay*

The phytotoxic ability of plants and their compounds against other plants is being investigated as an alternative to synthetic pesticides used for weed control in agricultural areas [41]. To evaluate the phytotoxic ability, crude and subfractions were used at varying doses from low to high. Crude methanol (DCMF) and aqueous (DCAF) fractions were found to have maximum and minimum activity, with 90% and 80% plant growth inhibition, respectively, at 1000 µg/mL. Similarly, chloroform (50.0%) and methanol (35.0%) extracts showed moderate activity at lower concentrations of 100 and 10 µg/mL, respectively. The activity remained the same (27.5%) in n-hexane (DCHF), chloroform (DCCF), and ethyl acetate (DCEF) at the lowest concentration, i.e., 10 µg/mL (**Table 3**). The plants contain some active constituents that have a phytotoxic effect, as stated by Rob et al. [41]. On the other hand, the genus *Datisca* contains cytotoxic triterpenes and flavonoids, which can cause host tissue death since these toxins can easily enter host cells [42-44]. Our findings concurred with the data reported by Hussain et al. [45] and Rashid et al. [32] for some selected plant species. Additionally, our outcomes

did not align with the observed data of Marona et al. [46] for *Ateleia glazioviana*. The variation in phytotoxic potential may be due to the quality and quantity of active ingredients available in plants, as documented by Bakhtiari et al. [47].

3.4. Insecticidal activity

The chloroform and ethyl acetate fractions of *D. canabinna* exhibited moderate insecticidal activity, while aqueous and butanol fractions revealed low activity against *Callosbruchus analis*. The remaining fractions exhibited no activity against T. castaneum, low activity against R. dominica, and moderate activity against C. analis (**Table 4**), compared to the standard drug (permethrin) and both positive and negative controls. Our results are in complete agreement with the reported data by Rashid et al. [32].

3.5. Antioxidant and urease bioassays

The free radicals formed in the human body have adverse effects that need to be neutralized using natural sources as an alternative, effective remedy [48, 49]. However, much attention has been focused on discovering innovative natural, potent antioxidants, including herbal products, intended to reduce the biologically harmful free radicals [50]. DPPH radicals have been widely used to evaluate the antioxidant properties and minimize adverse effects, as stated by Zhang et al. [51]. Keeping in mind the importance of antioxidants, the understudy plant was tested for free radical scavenging properties to validate local information and highlight a natural, novel, and effective cure for neutralizing free radicals and preventing cellular membrane damage. Among all the fractions screened, DCEF (86.0%) displayed the highest activity followed by DCCF fraction (75%), while DCMF (18%) attributed minimum activity compared to standard BHA (92.25%) at a concentration of 1.0 mg/ml. The active samples reduced the stable radical DPPH to the yellow-colored diphenyl picrylhydrazine (**Table 5**). The current findings were equivalent to Aliyu et al. [52] in vitro examination, which demonstrated the anti-oxidant properties of *Bauhinia rufescens* Lam leaf extracts.

Table 3. *In vitro* phytotoxic bioassay of the *D. canabinna*

Samples	Conc. (µg/mL)	No. of fronds samples	Control	% Growth regulation	Conc. of standard. Drug (µg/mL)
DCHF		15		82.5 ± 1.25	
DCCF		08		82.5 ± 2.12	
DCEF	1000	09	40	82.5 ± 1.27	0.015 ± 0.92
DCBF		04		85.0 ± 1.05	
DCAF		02		80.0 ± 0.98	
DCMF		05		90.0 ± 0.68	
DCHF		25		42.5 ± 1.81	
DCCF		27		50.0 ± 1.28	
DCEF	100	23	40	30.0 ± 1.12	0.015 ± 0.52
DCBF		24		45.0 ± 2.25	
DCAF		20		37.5 ± 1.85	
DCMF		23		40.0 ± 1.20	
DCHF		30		27.5 ± 1.12	
DCCF		28		27.5 ± 2.24	
DCEF	10	30	40	27.5 ± 1.54	0.015 ± 0.64
DCBF		29		25.0 ± 1.58	
DCAF		27		25.0 ± 1.34	
DCMF		29		35.0 ± 1.45	

Lemna minor was used to investigate the phytotoxic activity; Incubation condition: 28 °C; crude methanol extract (DCMF), n-hexane fraction (DCHF), chloroform fraction (DCCF), ethyl acetate fraction (DCEF), n-butanol fraction (DCBF), and aqueous fraction (DCAF); Conc. concentration; Std. Standard.

Table 4. Insecticidal activity of *D. canabinna* by the contact toxicity method.

Insect species	+ve control	-ve control	% Mortality					
			DCHF	DCCF	DCEF	DCBF	DCAF	DCMF
C. analis	100	0	20	50 ± 0.84	40 ± 0.54	20 ± 1.45	30 ± 0.65	10 ± 1.65
T. castaneum	100	0	NA	NA	10 ± 1.92	NA	NA	NA
R. dominica	100	0	NA	10 ± 1.24	10 ± 1.57	NA	NA	NA

+ive control: standard insecticide (Permethrin) and test insects; -ive control: volatile solvents and test insects; NA: not active; crude methanol extract (DCMF), n-hexane fraction (DCHF), chloroform fraction (DCCF), ethyl acetate fraction (DCEF), n-butanol fraction (DCBF), and aqueous fraction (DCAF); *T. castaneum = Tribolium castaneum, C. analis = Callosbruchus analis,* and *R. dominica = Rhyzopertha dominica*

Additionally, a study conducted by Al-Gburi et al. 2024 [53] determined the antioxidant potential of the methanolic extracts of *Caesalpinia volkensii* Harms. Furthermore, our study results are validated by those of Sowndhararajan et al. [54], who reported the antioxidant activity of Indian Acacia species methanolic bark extracts. Similar results were obtained by Al-Gburi et al. 2024 [53]. These findings also showed that DCEF could be utilized as a shield against human bodily harm due to its anti-free radical properties.

The urease enzyme serves a critical role in the continuous residence of Helicobacter pylori (H. pylori), which causes gastrointestinal disorders, particularly duodenal ulcers, gastritis, and gastric cancer [49]. In the case of enzyme inhibition studies, the methanol extract, along with its fractions from D. cannabina, was screened against the urease (jack bean) enzyme at a concentration of 1.0 mg/ml. The results indicated that DCEF (90%) exhibited the maximum inhibition, followed by DCBF (88%) and DCAF (70%), while the other fractions were found to be inactive (**Table 5**). These results have revealed that *D. cannabina* can be a source of natural inhibitors of this enzyme. Previously, nine compounds, namely datisdirin, tectochrysine, cearoin, sideroxyline, ursolic acid, corosolic acid, arjunolic acid, erythrodiol, and oleanolic acid, were isolated from the ethyl acetate fraction of *D. cannabina*. Among them, a flavonoid (Datisdirin) showed potent activity with an IC50 value of 83.79 ± 0.023 µM, compared to the standard thiourea (IC50, 21.01 ± 0.51 µM) [26], which further strengthened our current findings. A similar type of result is reported through the investigation of different medicinal plants against the urease enzyme [49, 53, 54]. Bai et al. (2015) investigated the urease inhibitory activity of fifteen crude extracts from Indian medicinal plants and their ability to inhibit bacteria causing urinary infections, reporting five active plant extracts at the same concentration used in the current study [55].

Table 5. Antioxidant and urease potential of the different fractions of *D. cannabina*

Tested Samples	% Inhibition (antioxidant)	% Inhibition (urease)
DCHF	26.0 ± 2.92	NA
DCCF	75.0 ± 1.88	NA
DCEF	86.0 ± 0.92	90 ± 0.92
DCBF	34.0 ± 1.38	88 ± 0.58
DCAF	25.0 ± 1.58	70 ± 1.32
DCMF	18.0 ± 1.92	NA
Standard (BHA)	91.25 ± 0.52	-
Thiourea	-	98.0 ± 0.52

Crude extract (DCMF), n-hexane fraction (DCHF), chloroform fraction (DCCF), ethyl acetate fraction (DCEF), n-butanol fraction (DCBF), and aqueous fraction (DCAF), Butylated hydroxyanisole (BHA).

Similarly, Biglar and his Colleagues (2014) screened 80% aqueous methanol extracts of twenty Iranian medicinal plants against urease and found that eight of the 20 studied plants' had the most effective crude extracts [49]. The DCEF and DCBF fractions of *D. cannabina* were found to have higher inhibition than these selected medicinal plants, which may be due to the different climate and environmental conditions. Our findings may help to explain the beneficial effect of this plant against infections associated with the urease enzyme. The DCBF fraction, in addition to DCEF, might also be an excellent source of compounds with good urease inhibitory activity if further investigated through bioassay-guided isolation.

3.6. Spasmogenic and spasmolytic activity

This experiment was conducted on an isolated rabbit intestine, and the spontaneous movements of the intestine were recorded on an Oscilloscope using an isotonic transducer (Harvard Isolated Organ Bath apparatus). The effects of crude extract and fractions were compared with standard drugs, i.e., acetylcholine, adrenaline, and atropine. The crude extract and fractions were administered at varying concentrations to determine the maximum effect at the lowest concentration (**Figures S1-S3**). The effects of crude extract and its fractions on the contraction and relaxation pattern of isolated rabbit intestines (smooth muscles) were recorded and presented below in tabular form (**Tables 5-6**). It is a sensitive evaluation method for drugs containing compounds that can act through receptors or act directly. In this regard, the following results were obtained. **Figure S1** displayed the tracing of smooth muscle activity of the crude extract of *D. cannabina*. The extract was run at different doses, including 1, 5, 10, 15, 20, and 25 mg/mL. The initial response of the drug at 1 and 5 mg/mL appears as a slight contraction in the rabbit intestinal tissue, whereas the doses of 10, 15, 20, and 25 mg/mL displayed low smooth muscle relaxation of the intestine. Upon separating different fractions of the crude extract, the effect of the drug is significant.

Table 6. Dose-related response of the methanol extract of *D. cannabina* on isolated rabbit intestine

Dose (mg/mL)	Control (cm) (SEM)	Response (cm) (SEM)	% Response	t- value
01	0.76 ± 0.12	0.73 ± 0.08	4	0.222
05	0.60 ± 0.05	0.73 ± 0.03	22	-2.049
10	0.70 ± 0.03	0.56 ± 0.03	19	4.240*
15	0.70 ± 0.02	0.4 ± 0.057	43	5.357*
20	0.76 ± 0.06	0.46 ± 0.27	40	0.4526**
25	0.83 ± 0.06	0.33 ± 0.60	60	0.584**

The results are expressed in cm ± SEM, at P < 0.05; * significant, **highly significant, SEM, standard error mean.

Figure S2(A) determined the effect of the ethyl acetate fraction of the *D. cannabina* on the drug. There is dose-dependent relaxation of muscle activity, whereas in the case of chloroform (**Figure S2(B)**), contraction was produced in the intestine. This effect was not observed in the crude methanol extract (**Figure S1**). The n-hexane fraction (**Figure S2(C)**) attributed a slight relaxation in the intestinal muscle. In contrast, *n*-butanol and aqueous fractions displayed a significant dose-dependent contraction of smooth muscles of the intestine. These fractions were further treated with standard drugs in different concentrations. Since the prominent effect was observed in ethyl acetate, chloroform, and aqueous fractions, these fractions were selected to explore the possible mechanism of action of the drug. **Figure S3** determined the tracing of smooth muscle activity of an aqueous fraction of *D. canabinna* with standard drugs on isolated rabbit intestines. Post-treated tissue with adrenaline conc. 1×10^{-4} M showed that the standard drug did not inhibit the effect of the drugs. When the same dose of the aqueous fraction is repeated with a high concentration of adrenaline, at a concentration of 1 × 10^ (-2)line that is 1 × 1the 0^ (-isM, the effect of the drug was also not inhibited (thus, there is a possibility of occupancy of the adrenergic receptor), when this aqueous fraction is pre- and post-treated with1×10^{-4} M conc. of acetylcholine (**Figure S3**), the contractile effect of acetylcholine was not produced. Similarly, in the case of ethyl acetate fraction pre- and post-treated tissue with acetylcholine at 1 × 10^ (-4) M, followed by administration of the drug, the effect of the drug was also not observed (**Figure S4a-n**, Supporting Information). From these results, there may be possible involvement of both adrenergic and muscarinic receptors. When the aqueous fraction is treated with 1 × 10^ (-2) M and 1 × 10^ (- 4) M concentrations of adrenaline, the effect of the drug is not observed, which means there is beta-adrenergic receptor blockage. The effect of acetylcholine 1×10^{-2} M was also not produced with a 15 mg/mL dose of *n*-butanol fraction (**Tables 6** and **7**).

3.7. Analgesic activity

Although synthetic medications are expensive and have many undesirable effects, plants are being examined as alternatives for evaluating analgesic drugs [15]. Research on medicinal plants, including their analgesic effects, is crucial for treating various ailments and challenges [16]. This activity was performed to evaluate the analgesic properties of the methanol extract from this plant, and the results are presented in **Table 8**. The test

was performed in comparison to standard drugs, and each experiment was repeated in triplicate. There was no significant difference in the thermal stimulus in mice treated with the vehicle (negative control) throughout the entire experiment. The doses of methanol extract were administered at 300 and 500 mg/kg. The best results were obtained at a 90-minute dose of 500 mg/kg and remained constant up to 120 minutes, after which they decreased gradually to 210 minutes. In the case of the 300 mg/kg dose, analgesia reached a maximum at 120 minutes and then decreased gradually. This means that a higher dose is more effective compared to a low dose, while the duration of pain relief is short, and the effect remains constant. The overall results of the methanol extract in terms of analgesic activity were found to be excellent and statistically significant.

Our results are in complete agreement with the ethanolic extract of *Papaver libanoticum* using hot plate-induced writhing models in mice [56]. Recently, Ayanaw et al. (2023) reported the analgesic activity of the methanolic extract of *Gomphocarpus purpurascens* in Mice [57]. Another study, conducted by Shojai et al. (2015), examined the different extracts of Astragalus hamosus pods, a plant used in Iranian traditional medicine, using the acetic-acid-induced writhing response and hot plate test. The results of their study demonstrated the analgesic effects of hydro-alcoholic extract in animal models [58]. A similar type of finding was reported in the literature [59-62]. The latest findings align with the herb's traditional use in treating painful and chronic inflammatory conditions. However, the exact methods by which *D. cannabina* exerts analgesic properties remain unknown. Further research is needed to determine the specific mechanism underlying the use of this plant in pain treatment.

Table 7. Effects of different fractions of *D. canabinna* on isolated rabbit intestine

Fractions	Dose (mg/mL)	Control (cm) (SEM)	Response (cm) (SEM)	% Response	t- value
DCEF	05	0.80 ± 0.057	0.53 ± 0.032	33.37	4.120
	10	0.80 ± 0.00	0.43 ± 0.032	45.87	11.468*
	15	0.86 ± 0.032	0.40 ± 0.00	53.48	14.5625*
DCCF	05	1.03 ± 0.032	0.36 ± 0.032	64.46	15.09**
	10	0.83 ± 0.032	1.16 ± 0.032	39.25	-7.43
	15	0.70 ± 0.057	1.36 ± 0.032	95.14	-10.27**
DCHF	05	1.00 ± 0.00	0.93 ± 0.032	6.70	2.09
	10	0.90 ± 0.00	0.73 ± 0.066	18.55	2.53
	15	0.83 ± 0.032	0.53 ± 0.032	36.14	6.818
DCBF	05	0.90 ± 0.00	0.66 ± 0.087	26.66	2.689
	10	0.66 ± 0.032	1.20 ± 0.099	81.81	-5.242**
	15	0.66 ± 0.087	1.06 ± 0.066	60.60	-3.66**
DCAF	05	0.80 ± 0.00	0.83 ± 0.066	-4.125	-0.5
	10	0.96 ± 0.032	1.36 ± 0.087	40.78	-4.28
	15	0.93 ± 0.032	1.43 ± 0.032	53.26	-11.29*

Crude methanol extract (DCMF), n-hexane fraction (DCHF), chloroform fraction (DCCF), ethyl acetate fraction (DCEF), n-butanol fraction (DCBF), and aqueous fraction (DCAF). Results are expressed in ± SEM, at $P < 0.05$ *significant, **highly significant. SEM, standard error mean.

Table 8. Analgesic significance of the methanol extract of *D. cannabina* on the hot plate method.

Parameter	Time in minutes							
	0	**30**	**60**	**90**	**120**	**150**	**180**	**210**
Control	2.98 ± 0.44	1.8 ± 0.37	2.4 ± 0.24	1.8 ± 0.24	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.6 ± 0.2
Diclofenac sodium (50 mg/kg)	1.97 ± 0.13	2.6 ± 0.25	3.6 ± 0.25	4.12 ± 0.09	4.06 ± 0.09	3.3 ± 0.19	2.3 ± 0.19	2.1 ± 0.14
Treated (300 mg/kg)	1.6 ± 0.24	3.0 ± 0.25	3.0 ± 0.25	3.6 ± 0.18	4.2 ± 0.22	3.3 ± 0.2	2.7 ± 0.2	2.5 ± 0.2
Treated (500 mg/kg)	2.0 ± 0.23	3.6 ± 0 .24	4.6 ± 0.24	5.0 ± 0.5	5.0 ± 0.5	4.0 ± 0.45	3.3 ± 0.12	3.0 ± 0.43

4. Conclusion

In the current investigation, various biological activities, including analgesic, antioxidants, phytotoxic, cytotoxic, hemagglutination, urease, and spasmodic and non-spasmodic activities of D. Cannabina, were evaluated. The ethyl acetate fraction exhibited excellent inhibition in both antioxidant and urease assays. Similarly, the crude methanol extract displayed significant analgesic potential compared to the other tested samples and the standard, indicating the presence of bioactive chemical constituents. All extracts showed a high or medium agglutination effect against all human blood groups at a high concentration, except for blood groups B and A, where the effect was low. Moderate to mild agglutination activity at high concentrations suggested the presence of a lectin in trace amounts. The methanol and aqueous fractions exhibited a maximum phytotoxic activity of 90% and a minimum of 80% at higher concentrations only. Higher phytotoxicity indicated a strong protection mechanism against herbivores or insects. The pharmacological action on smooth muscles is suggestive of the mode of action of the crude extract through adrenergic and muscarinic receptors. Based on the above facts, it is concluded that D. cannabina may be a valuable source of medicine, and further research is required to isolate the pharmacologically active phytochemicals.

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Conflict of interest

The authors declare no conflict of interest regarding the submission of this manuscript.

Data availability

All datasets on which the conclusion of the manuscript relied are presented in the paper.

Authors Contributions

Zainab did collection and fractionation of the plant species. AA performed antioxidant and urease activities. NM, and Mehjabeen accomplished spasmogenic and spasmolytic activity, while Mehjabeen managed brine shrimp lethality bioassay. NJ and MS performed phytotoxic, hemagglutination and insecticidal activities. NUR analysed the data and wrote the original draft of the manuscript. MA, and AA-H supervised the project, assisted in the writing, the reviewing, and the editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

Authors Orcid numbers:

Abdul Aziz: 0000-0002-6034-7255
Muddaser Shah: 0000-0001-8839-4419
Noor Jahan: 0000-0002-7286-3177
Najeeb Ur Rehman: 0000-0002-1563-225X
Ahmed Al-Harrasi: 0000-0002-0815-5942
Manzoor Ahmad: 0000-0001-9736-5900

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Using artificial intelligence chatbots

There was no use of artificial intelligence in the making of this article.

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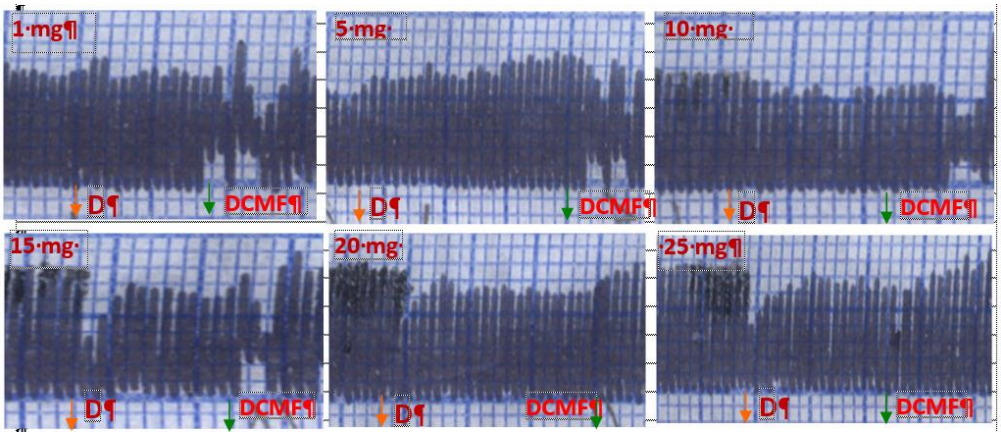

Figure S1. Tracing of smooth muscle activity of methanol extract of *D. cannabina* with drug (D).

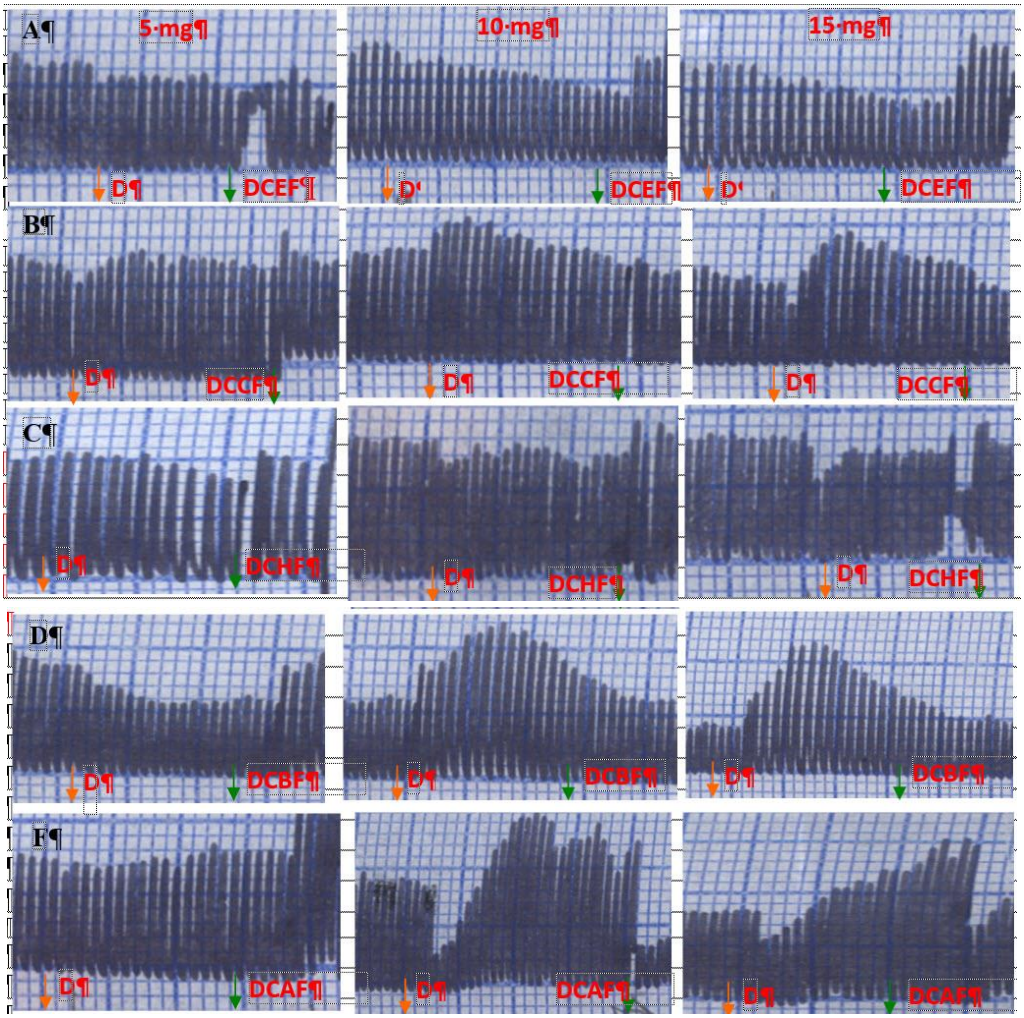

Figure S2. Tracing of smooth muscle activity of different fractions of *D. cannabina* and drug.; D, drug using doses of 5, 10, and 15 mg/mL; **A**) DCEF, ethyl acetate fraction; **B**) DCCF, chloroform fraction; **C**) DCHF, n-hexane fraction; **D**) DCBF, n-butanol fraction; **E**) DCAF, aqueous fraction; Ad, adrenaline; Ach, acetylcholine.

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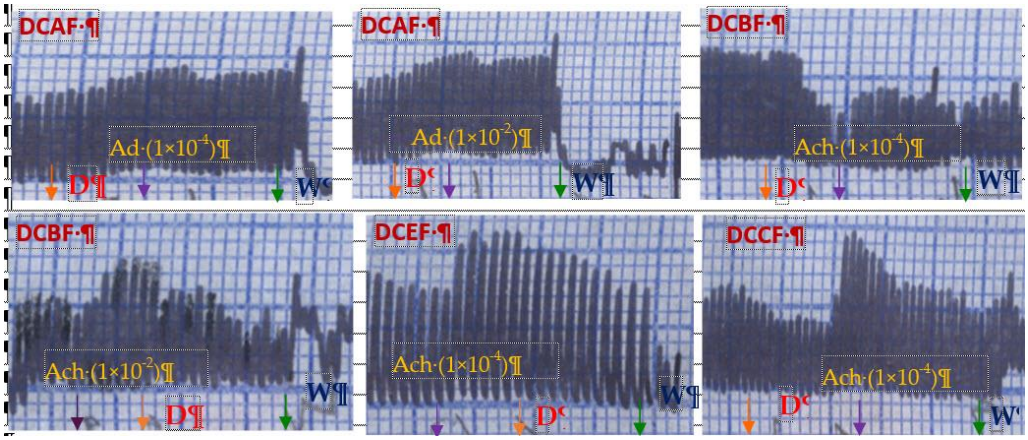

Figure S3. Tracing of smooth muscle activity of different fractions of *D. cannabina* with standard drug on isolated rabbit intestine using doses of 15 mg/mL; D, drug; W, water; DCCF, chloroform fraction; DCEF, ethyl acetate fraction; DCBF, n-butanol fraction, and DCAF, aqueous fraction; Ad, adrenaline; Ach, acetylcholine.

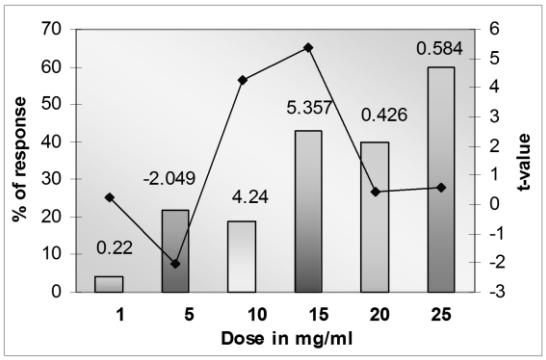

Figure S4a: The *in vitro* experiments showing % response of DCMF of *D. canabinna* on isolated intestine rabbit

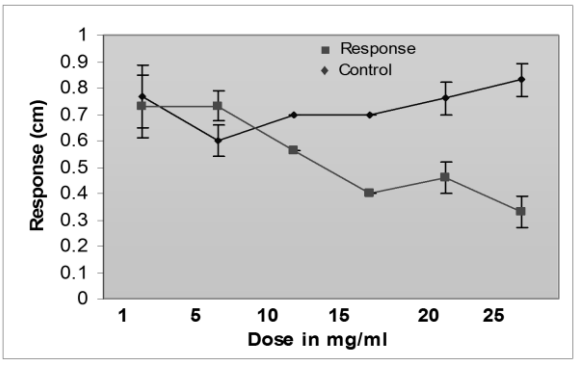

Figure S4b: The *in vitro* experiments showing the dose response (cm) of DCMF of *D. canabinna* on isolated intestine rabbit.

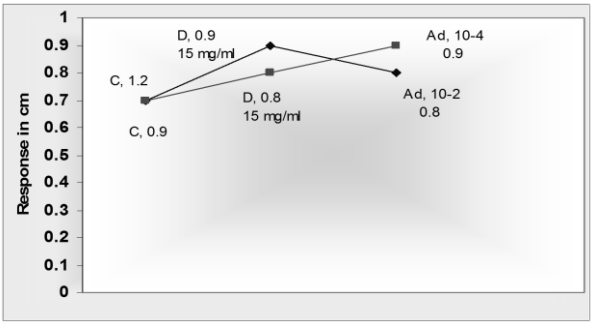

Figure S4c: Effect of DCAF of *D. cnanbinna* on post-treated with adrenaline 1×10^{-4} M and adrenaline 1×10^{-2} M concentration

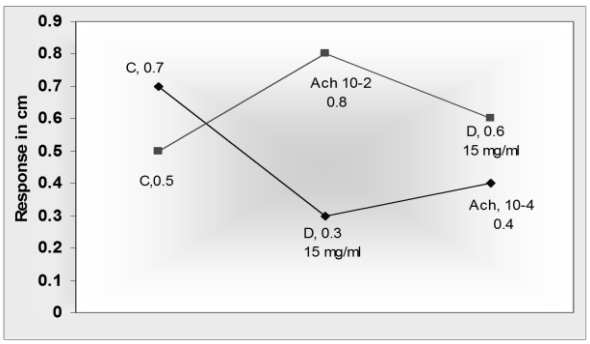

Figure S4d: Effect of DCBF fraction on pre- and post-treated with acetylcholine 1×10^{-4} M concentration

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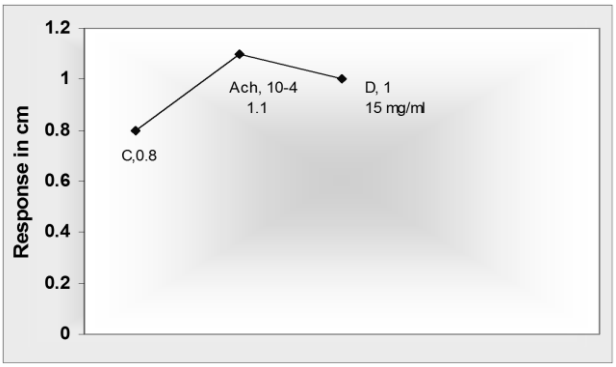

Figure S4e: Effect of DCEF on pre- treated with acetylcholine 1×10^{-4} M concentration

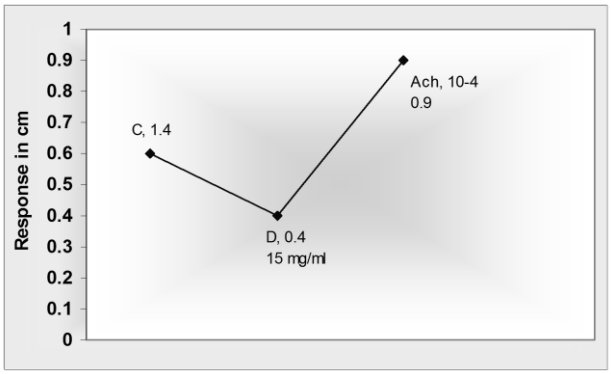

Figure S4f: Effect of DCCF on post-treated with acetylcholine 1×10^{-4} M concentration

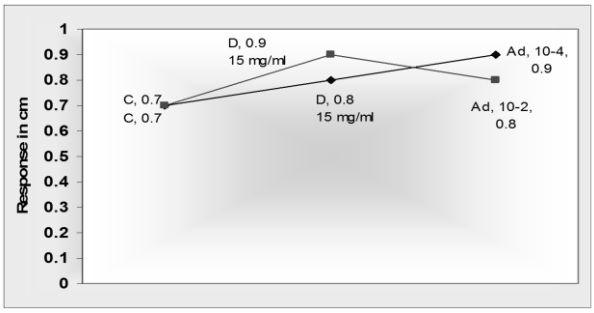

Figure S4g: Effect of DCAF on pre- and post- treated with adrenaline 1×10^{-4} M and 1×10^{-2} M concentration

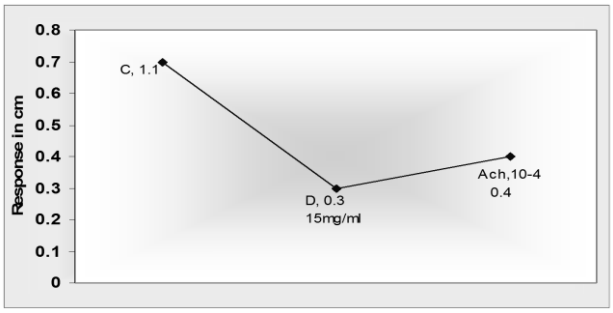

Figure S4h: Effect of DCBF on post-treated with acetylcholine1×10^{-4} M concentration

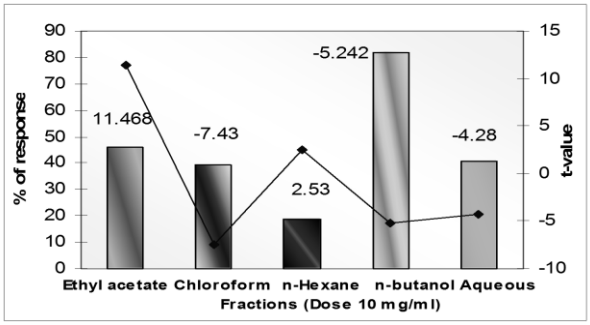

Figure S4i: % response of different fractions on isolated intestine of rabbit at 10 mg/mL

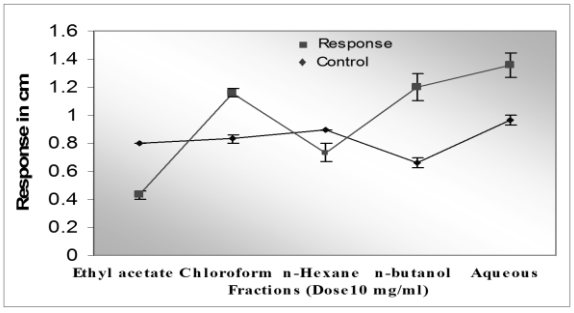

Figure S4j: Dose response along with SEM of different fractions on isolated intestine of rabbit at 10 mg/mL

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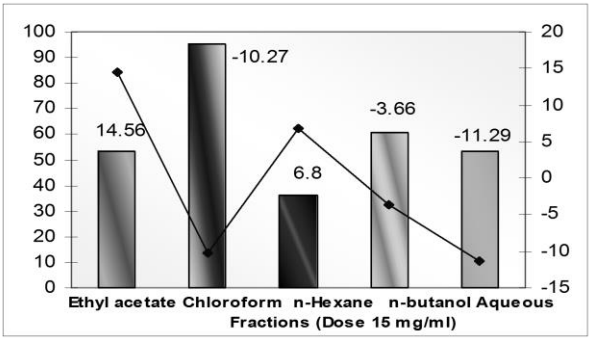

Figure S4k: % response of different fractions on isolated intestine of rabbit at 15 mg/mL

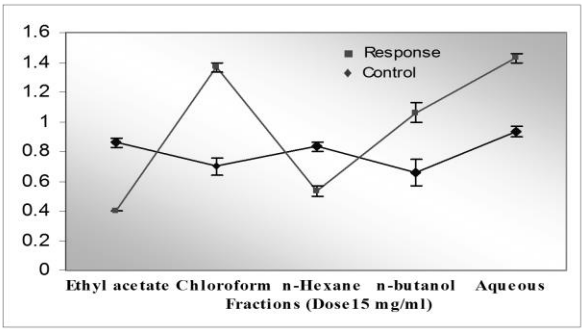

Figure S4l: Dose response along with SEM of different fractions on isolated intestine at 15 mg/mL

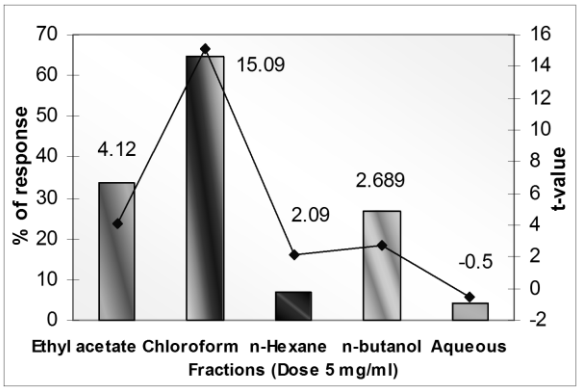

Figure S4m: % response of different fractions on isolated intestine at 5 mg/mL.

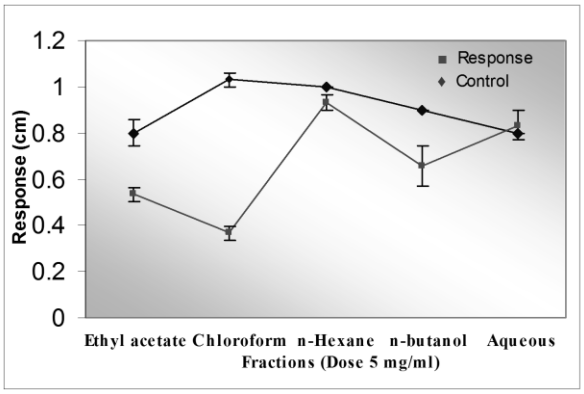

Figure S4n: Dose response along with SEM of different fractions on isolated intestine at 5 mg/mL.

Figure S4a-n: Effect of different fractions of *D. cnanbinna* on isolated intestine rabbit and pre- and post-treated with adrenaline 1×10^{-4} M and adrenaline 1×10^{-2} M concentration