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RESEARCH ARTICLE

LC-MS-Based Phytochemical Analysis and Anthelmintic Potency of *Cassia fistula* (Linn.) Extracts Against Gastrointestinal Nematodes of Sheep and Goats

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Abstract

Medicinal plants play an important role in addressing global health challenges in both humans and livestock, particularly those associated with gastrointestinal parasitic infections. The present study aimed to conduct comprehensive LC-MS-based phytochemical profiling of various *Cassia fistula* extracts and their anthelmintic activity against gastrointestinal parasites in sheep and goats. *In vitro* anthelmintic efficacy of crude extracts prepared from different plant parts (leaves, stem, fruit, and root) was evaluated using two-fold serial dilutions, with 50 mg/mL as the stock concentration. Levamisole, Oxfendazole, and Albendazole were used as positive controls, and phosphate-buffered saline (PBS) as the negative control. Based on *in vitro* performance, the ethanol fruit extract and crude fruit powder were further assessed *in vivo* in goats (n=40) at dose rates of 250, 500, and 1000 mg/kg body weight. Phytochemical characterization of all extracts was carried out using LC-MS analysis under both positive and negative electrospray ionization modes. Treatment with the crude ethanol fruit extract against *Haemonchus contortus*, resulted in mortality of all adult parasites within 4 h and 90.33% inhibition of egg hatching at 50 mg/mL, which was comparable and significantly higher than the positive controls ($P<0.05$). In case of the *in vivo* study at Day 21, crude fruit ethanol extract with the highest dose (1000 mg/kg) significantly reduced parasite burden of 90.78±0.55%, which was comparable to ABL (87.91±2.08%) ($P>0.05$). A total of 220 compounds were identified in LC-MS analysis of *C. fistula* including anti-parasitic constituents; quercetin, kaempferol, gallic acid, and hydroxycinnamic acid derivatives. These findings suggest that *C. fistula* fruits possess notable anthelmintic potential as a natural alternative to synthetic anthelmintics. However, further studies, including detailed toxicity assessments and dose optimization, are required to establish their safety and therapeutic applicability in livestock.

Keywords: Anthelmintic efficacy, *C. fistula*, Gallic acid, *H. contortus*, LC-MS analysis

INTRODUCTION

Livestock growth and productivity are adversely impacted by the economic burden of gastrointestinal parasites, which lead to substantial losses through reduced growth, shortened lifespan, infertility, and decreased survival rates [1]. Among gastrointestinal helminth infections, haemonchosis holds major global economic significance, as it restrains the fecundity and the sustainability in small ruminant populations [2]. This disease affects the host's immune function, hematological profile, and biochemical parameters, ultimately resulting in reduced digestion, constant blood loss, protein depletion, and eventually causing host death [3]. The primary causative agent of Haemonchosis, *Haemonchus contortus* (*H. contortus*), cause significant economic loss to livestock

across temperate and tropical zones, estimated to reach billions of dollars annually to the veterinary market [4]. It is considered one of the most resistant parasitic species due to its high pathogenicity and prolific reproductive capacity [5].

Parasite control has traditionally relied on synthetic anthelmintics, along with dietary and grazing management strategies [6]. However, synthetic anthelmintics are often associated with toxicity issues and adverse effects, including gastrointestinal disturbances, hepatotoxicity, neurotoxicity, and reduced productivity, as well as high costs and limited availability [7]. Moreover, the multi-resistant veterinary parasite, *H. contortus*, has developed resistance to nearly all widely used anthelmintic classes, including imidazothiazoles, ivermectin, benzimidazoles, and thiabendazole [8]. Therefore, Plant-derived anthelmintics are particularly promising because they tend to be less toxic, widely accessible, cost-effective, and easy to prepare and administer [9]. Nature offers a diverse range of bioactive compounds in plants, such as alkaloids, flavonoids, terpenoids, amino acids, sugars, and polyacetylenes that serve as defense mechanisms against bacteria, fungi, pests, viruses, and insects [10]. Moreover, the use of medicinal plants for managing parasitic infections has a long-standing historical association with both livestock and human health [11].

Advanced standardizing techniques are increasingly employed to identify secondary metabolites in plants and to evaluate their anthelmintic potential [12]. Liquid chromatography mass spectrometry (LC-MS) serves as a crucial analytical tool for the identification, quantification, and mass study of a wide spectrum of semi-volatile and volatile organic and inorganic compounds in a complex mixture [13]. The anthelmintic efficacy of medicinal plants, including different parts of the same plant, varies depending on their bioactive constituents, as well as factors such as geographical origin, cultivar, seasonal variations, and the solvents used for extraction [11].

Cassia (C.) *fistula* L. (commonly known as Amaltas or Golden shower) is an angiosperm plant belonging to the family Fabaceae. It is an average-sized ornamental tree characterized by its yellow cheerful flowers and dark green compound leaves [14]. In the Cholistan desert, local nomadic communities known as Roohelay traditionally used this tree as a medicinal and therapeutic moderator to support their livestock's health, growth, and productivity [15]. Previous findings have reported the anthelmintic activity of *C. fistula* leaves, where ethanol and n-hexane extracts demonstrated inhibitory effects against L3 infective larvae of *H. contortus* in larval migration inhibition assay [16]. *C. fistula* fruits may offer a rich reservoir of phyto-constituents possessing significant anthelmintic potential; hence, they may offer a promising natural alternative to commercial drugs for the proper control and management of gastrointestinal parasitic diseases.

Material and Methods

Ethical Approval

Animals experiments were conducted in strict adherence to the the guidelines for the Welfare and Ethical Review committee of Laboratory Animals as well as Institutional Animal Care and Use Committee (IACUC).The experiments were conducted following approval from the Animal Ethics' Committee of the Islamia University of Bahawalpur (Approval # 456.ORIC). The university-appointed research reviewers evaluated all relevant ethical considerations and confirmed that the study procedures adhered to established animal welfare standards.

Plant Parts Collection and Handling

C. fistula fruits, fresh roots, and leaves with attached soft stem branches (about 2kg) were collected between January and February 2023, from the Cholistan desert area of the Islamia University of Bahawalpur, Baghdad campus, Punjab, Pakistan. Then, the plant samples were taxonomically identified and authenticated as *C. fistula* (voucher no. 18_01_025) at the botany department of agriculture university, Faisalabad, Pakistan. The collected plant samples were separated and thoroughly washed with fresh water to remove dust and contaminants. They were then shade-dried at a room temperature of 25°C, chopped, and ground into coarse particles using a mechanical grinder.

Extract Preparation of *C. fistula*

The plant collected samples were sequentially extracted in ethanol, methanol, aqueous methanol, and ethyl acetate following slight adjustments [17,18]. The selection of these solvents was based on previously reported literature demonstrating that solvent polarity plays a critical role in the efficient extraction of diverse phytochemical classes [19]. In particular, polar solvents such as methanol and aqueous methanol are effective for extracting polar compounds (e.g., flavonoids, glycosides), while ethanol and ethyl acetate facilitate the extraction of moderately polar to semi-polar bioactive constituents, thereby ensuring a broad phytochemical profile relevant to biological activity [19].

Approximately 100 g of powdered material from each plant part was soaked in 400 mL of ethanol in a sealed container and intermittently stirred over three days. Then, filtration of the mixture was done, and the remaining plant residue was re-soaked in another 400 mL of ethanol. This process was repeated two additional times. Then, all filtrates were mixed, and the solvent was evaporated under reduced pressure at 40°C in a rotary evaporator. The resulting crude ethanolic extract, obtained in syrup form, was stored at 4°C until further study. The same procedure was

reiterated to prepare extracts in ethyl acetate, methanol, and 75% aqueous methanol (methanol-water ratio, 3:1).

Liquid Chromatography and Mass Spectrometry (LC-MS) Study

LC-MS analysis was performed following the method described by Kaur et al.[20]. All analyses were carried out using advanced instrumentation at International Laboratories of Textile Testing (TTI), Lahore, Pakistan. The chromatographic method employed was a water eluent in UPLC H-Class, prepared with a BEH-C18 column (2.1 × 100 mm, 1.7 µm particle size). This UPLC platform combines the sensitivity, high speed, and resolution of UPLC with the operational flexibility of conventional HPLC. The mobile phases (each of 0.1%) consisted of formic acid in water (mobile phase A) and formic acid in acetonitrile (mobile phase B) with a flow rate of 0.25 mL per minute. A gradient elution profile was applied to optimize compound separation, with the following solvent ratios (Time - A/B): 0 min –90/10, 2 min –90/10, 5 min –80/20, 10 min –70/30, 12 min –50/50, 14 min –90/10. A sample volume of 4 µL was injected into the column, and the column temperature was maintained at 30°C. The injection volume was kept constant across all analyses to ensure methodological consistency. This low volume of sample was used to prevent peak broadening and to maintain high resolution, which is critical for narrow-bore UPLC columns.

Mass spectrometric detection was conducted in a TOF-MS (time of flight mass) system equipped with an electrospray ionization (ESI) source. Instrument parameters included: cone gas and desolvation gas flows of 30 and 850 L/h (nitrogen/argon), respectively, desolvation and source temperature (450 and 120°C), capillary and cone voltage (3.21 kV and 50V), respectively, and collision energy of 4eV. Each extract was analyzed in both negative (-) and positive (+) ionization modes. All LC-MS analyses were performed using a Waters UPLC-QTOF MS system (Waters Corporation, Milford, MA, USA), ensuring methodological reproducibility. Plants' phytochemicals identification was based on LC retention time and accurate mass measurements. When available, reference standards were used for compound identification by matching both exact mass and retention time. However, the tentative identifications were made using accurate mass data, published literature, and characteristic fragmentation patterns of well-known metabolite classes.

Anthelmintic Assessment of Plant Extracts and Positive Control Groups

Adult Worms' Motility Inhibition Assay

The basic methodology of adults' motility inhibition assay was carried out following the method with slight adjustments [17,18]. The *in vitro* assays were conducted using a well-characterized and biologically relevant model parasite, *H. contortus*. This parasite species was specifically selected because it is recognized as one of the most prevalent and highly pathogenic gastrointestinal nematodes affecting small ruminants worldwide, including in our region. The active, energetic, and mature worms of *H. contortus* were collected instantly after evisceration from the abomasum of a naturally infected, freshly slaughtered sheep from a local slaughterhouse and transferred into phosphate-buffered saline solution (PBS). Actively motile adult worms (n=10) were placed into separate Petri dishes containing various plant extract concentrations (50, 25, 12.5, 6.25, 3.125 mg/mL), along with a negative control treatment (PBS; 20 mL per dish) and a positive control assay (Levamisole; 0.55 mg/mL). Levamisole was used in the adult motility inhibition assay because it is a well-established reference drug for evaluating neuromuscular paralysis in adult nematodes. The different doses of various plant extracts were prepared through two-fold serial dilutions in PBS, using 50 mg/mL as the stock concentration.

All assays were executed in triplicate and repeated three times at 25°C. The worm's motility was evaluated under an inverted microscope following 2 h intervals for a duration of 12 h to determine the anthelmintic activity of *C. fistula* extracts. Worms exhibiting no movement at the head or tail, were transferred to lukewarm saline for approximately 5 min to confirm their motility inhibition. The percentage worm's motility was measured by following the formula described by Resendiz-Gonzalez et al.[21].

Egg Hatch Assays

Egg hatch inhibition assays were conducted following the process of "World Association for the Advancement of Veterinary Parasitology (WAAVP)," with minor adjustments [17]. The mature female worms of *H. contortus* were recognized based on their larger size (18-30 mm) and characteristic reddish appearance due to the white uterus spiraling around the red gut, giving the typical barber-pole pattern. Fully mature females were washed with PBS solution and then homogenized in a mortar containing 5 mL of PBS to release the eggs. The resulting suspension was filtered through an 80-µm mesh sieve under gentle gravity (without applied pressure) to remove tissue debris, and no fixed duration was required for this step. The filtrate was then diluted with PBS to obtain an egg suspension of approximately 200 eggs/mL.

All the treatments were tested in triplicate and repeated thrice in 24 multi-well plates using two-fold serial dilution starting from 50 mg/mL stock solution (50, 25, 12.5, 6.25, and 3.125 mg/mL) for plant extracts and 50 µg/mL stock solution for positive control, Oxfendazole. A negative

control, PBS were included in the assay. Oxfendazole was selected for the egg hatch assay due to its strong ovicidal activity and its frequent use as a standard control in egg hatch inhibition studies. Oxfendazole concentrations were taken in µg/mL, and plant extract doses in mg/mL. The eggs' incubation in labeled plates was carried out at 70% relative humidity and 27°C for 48 h. After incubation, unhatched eggs and hatched larvae of either live or dead were recorded under a compound microscope using 40× magnification power. The percentage egg hatch inhibition rate was determined by applying the formula described by Dhruw et al.[22].

In Vivo Bioassays

In vivo anthelmintic evaluation was conducted using only the *C. fistula* fruit powder and ethanol fruit extract, as the fruit ethanol extract demonstrated the highest efficacy during the *in vitro* screening. For extract preparation, 1 kg of fruit powder was immersed in 3 L of ethanol, intermittently stirred, and then filtered through a spongy cloth. The same procedure was reiterated thrice, the filtrates were mixed, and then concentrated in a rotary evaporator. The resulting syrup form extract was stored at 4°C. *The in vivo* assessment was subsequently carried out using the method followed by Flores-Prado et al.[23].

Experimental Design

For the *in vivo* study, goats (n=40) of either sex, male and female, and naturally infected with mixed gastrointestinal parasitic species for over six months, were selected randomly. Consequently, the experimental animals were not artificially infected with a single parasite species, but instead represented a realistic field scenario where multiple nematode species coexist and contribute collectively to the parasite burden. All the animals had a weight between 16 and 25 kg and were aged 7-10 months. Throughout the study, they were maintained on an adequate supply of food, fodder, and concentrates. The goats were randomly assigned to eight groups of five animals each. Group 0 (T_0) served as the untreated control. Group 1 (T_1) was treated with a single oral dose of albendazole (10 mg/kg b.wt). Albendazole was chosen as the positive control because it is widely used in field conditions for the treatment of gastrointestinal nematodes in small ruminants and thus served as a practical therapeutic benchmark. Ethanol fruit extract was administered to groups 2, 3, and 4 (T_2, T_3 & T_4) correspondingly at dosage rates of 250, 500, and 1000 mg/kg b.wt. While the groups 5, 6, and 7 (T_5, T_6, T_7) were treated with fruit powder at the same corresponding dose levels (250, 500 & 1000 mg/kg b.wt.). All the enrolled goats exhibited substantial gastrointestinal nematode burden, as verified using the McMaster fecal eggs calculation method.

Fecal samples from each goat's rectum were collected usually in the morning, starting from the day before treatment (D_0) to post treatment days (D_7, D_{14} & D_{21}). For egg count assessment, approximately 2 g of feces were mixed in 30 mL of tap water and homogenized, then mixed with 30 mL of PBS solution. A small portion of this mixture was transferred to a McMaster counting chamber using a pipette and studied under a microscope at 10× magnification to evaluate the number of nematode eggs. Accordingly, Belga et al.[24] calculated the parasite egg reduction.

Statistical Analysis

The data recorded in both bioassays, in vitro and *in vivo* was analyzed using one-way ANOVA in SPSS. Significant differences among treated and untreated groups were determined using the Duncan multiple range test.

Results

Percentage of the Extracts' Yield

The solvent extraction of various extracts of *C. fistula* produced the following yields: fruit's ethanol, methanol, ethyl acetate, aqueous-methanol extracts (12.5, 13, 9.2, 8%), roots ethanol, methanol, aqueous-methanol, ethyl acetate extracts (6.5, 9, 7.4, 8.5%), stem ethanol, methanol, ethyl acetate, aqueous-methanol extracts (9.8, 7, 9.1,7.5%), leaves ethanol, methanol, ethyl acetate, aqueous-methanol extracts (11, 11.5, 7.5, 7.5 %) respectively. Among all extracts, the highest yield was obtained from the fruit's methanol extract, whereas the lowest yield was observed for the root ethanol extract. In case of the *in vivo* study, the ethanol fruit extract produced a yield of 14.25%. The extract percentage yield was calculated as the dried extracts' weight divided by the initial plant powders' weight, multiplied by 100 (% w/w *100).

Liquid Chromatography and Mass Spectrometry (LC-MS) Study of *C. fistula*

LC-MS profiling of extracts from different parts of *C. fistula* identified numerous major classes of pharmaceutical compounds, including alkaloids, glycosides, terpenoids, phenols, and flavonoids. The active pharmaceutical constituents detected in only trace amounts in the leaves extracts were excluded from *Table 1*. Adicardin, myricetin, adenosine diphosphate, phytol sodium, lauric acid, pyroglutamic acid were present in trace amounts in leaves ethanol extract; cycloartenol acetate, catalpol sodium, succinylproline, 3-indoleacrylic acid in leaves methanol extract; myricetin, arachidonic acid, cycloartenol acetate, vomifoliol, dihydroquercetin in leaves ethyl acetate extract; quercetin-3′-O-glucoside, coniine, levulinic acid in aqueous-methanol extract. Similarly, compounds present in minimal quantities in the stem extracts were not included in *Table 2*. These included campesterol, propyl gallate,

Table 1. *Phytochemical compounds detected in LC-MS study in different C. fistula leaves' extracts*

Phyto-Constituents		Class Name	R.T. (min.)	% Area	M.W. (g/mol)	% Abund.
Bioactive metabolites present in leaves' ethanol extract	Quercetin	Flavonoids	2.75	1.89	302.2	3.33
	Proanthocyanidin B-dimers'	Flavonoids	5.31	4.14	578.5	1.14
	Vicenin-2	Flavonoids	0.69	2.13	594.5	2.11
	Gallic Acid	Phenolic Acids	1.59	1.23	170.12	1.06
	Apigenin-6C-Pentoside-8C-hexoside (isomer I)	Flavonoids	1.81	1.54	564.5	5.30
	Syringaresinol	Lignan	1.52	5.31	418.4	2.97
	Coumaric Acid	Phenolic Acids	2.91	5.75	308.5	1.67
	Ferulic Acid	Phenolic Acids	6.77	7.93	194.2	3.04
	Myricetin Hexoside	Flavonoids	1.79	7.46	480.4	3.33
	Kaempferol Rhamnosyl Xyloside	Flavonoids	3.34	4.24	564.5	1.73
	3,4-Di-O-Caffeoylquinic Acid	Phenolic Acids	3.48	2.68	516.5	1.06
	Astragalin	Flavonoids	1.11	1.21	448.4	1.96
	Protocatechuic Acid-4O-glucoside	Phenolic Acid	0.82	1.31	316.3	1.73
Bioactive metabolites present in leaves' methanol extract	Kaempferol 3O-Acetyl-Glycoside	Flavonoids	6.21	1.21	490.4	1.96
	p-Coumaric Acid	Phenolic Acids	3.49	2.56	164	2.90
	Vitexin	Flavonoids	1.52	3.44	432..4	1.60
	Rutin	Flavonoids	1.52	6.40	610.5	1.25
	Alpha-Linolenic Acid	Fatty Acids	2.31	3.53	278.4	4.04
	Apigenin-6C-Pentoside-8C-hexoside (isomer II)	Flavonoids	0.81	5.91	564.5	2.84
	Quercetin-3O-Hexoside	Flavonoids	7.71	6.05	463.4	2.99
	Neochlorogenic Acid	Polyphenols	5.29	4.88	354.3	1.46
	Quercetin 3O-2Acetyl-Glucoside	Flavonoids	2.91	6.71	506.4	7.19
	Syringaresinol	Lignan	6.57	6.64	418.4	3.91
	Apigenin-6C-Pentoside-8C-hexoside (isomer III)	Flavonoids	2.67	9.39	564.5	2.68
	Isorhamnetin-3O-Glucoside	Polyphenols	6.01	5.53	478.4	1.86
	Isorhamnetin	Flavonoids	8.37	7.42	316.2	6.27
Bioactive metabolites present in leaves' ethyl acetate extract	Vicenin 2	Flavonoids	2.25	7.47	564.5	4.04
	Trigonelline	Alkaloids	5.53	8.17	137.1	2.14
	Kaempferol Rhamnosyl Xyloside	Flavonoids	4.80	3.34	564.5	3.07
	L-Valine	α-Amino Acids	6.04	4.14	117.1	4.54
	Apigenin-6C-pentoside-8-Chexoside (isomer II)	Flavonoids	6.47	1.36	564.5	4.52
	p-Coumaric Acid	Phenolic Acids	3.81	2.31	308.5	2.10
	3,4-Di-O-Caffeoylquinic Acid	Phenolic Acids	1.91	1.67	516.5	6.21
	Myricetin Hexoside	Flavonoids	1.09	1.73	480.4	3.65
	Proanthocyanidin B-dimers'	Flavonoids	2.48	1.14	578.6	2.63
	Quercetin 3O-Hexoside	Flavonoids	4.71	3.05	463.4	5.00

Table 1. *Continue*

Phyto-Constituents		Class Name	R.T. (min.)	% Area	M.W. (g/mol)	% Abund.
Bioactive metabolites present in leaves' aqueous methanol extract	Apigenin-6,8-Di-C- Glycoside	Flavonoids	5.51	3.06	564.5	3.11
	Apigenin-6C-Pentoside-8-Chexoside (1st isomer)	Flavonoids	1.75	1.26	564.5	4.48
	Apigenin-C-Hexoside-O-Pentoside	Flavonoids	2.05	1.48	270.2	1.88
	Apigenin-6C-pentoside-8C-hexoside (2nd isomer)		1.24	2.33	564.5	1.65
	Trigonelline	Alkaloids	1.70	1.61	137.1	2.67
	Vicenin-2	Glycosides	2.11	1.22	594.5	8.53
	3,4-Di-O-Caffeoylquinic Acid	Phenolic Acids	1.92	1.40	516.5	2.68
	Isoquercitrin	Flavonoids	1.74	4.11	464.4	4.90
	Kaempferol-3O-Rutinoside	Glycosides	1.61	1.83	594.6	2.02
	Quercetin 3O-Hexoside	Flavonoids	2.61	1.24	463.4	1.44
	Kaempferol	Flavonoids	1.78	1.61	286.2	2.30

RT: Retention time, MW: Molecular weight, abund.: abundance

prenylated chalcone in ethanol extract; myrin succinyl, myricetin-3-O-xyloside, vanillic acid 4-sulfate, rutin acetate in methanol extract; asparagine, leucyl alanine, phytol in ethyl acetate extract; caffeine, pterostilbene, pallidol malonyl in aqueous-methanol extract.

A total of 14 phyto-constituents were identified in the root ethanol extract (1 phenol, 2 flavonoids, 3 phenolic acids, 4 terpenoids, 1 amino acid, 2 fatty acids, 1 stilbenoid), 13 in root methanol extract (1 amino acid, 1 steroid, 1 alkaloid, 1 glycoside, 4 flavonoids, 1 phenolic acid, 2 terpenoids, 2 polyphenols), 14 in root ethyl acetate extract (1 alkaloid, 2 flavonoids, 1 amino acid, 2 fatty acids, 4 phenols, 3 terpenoids), 14 in aqueous methanol extract (1 alkaloid, 3 flavonoids, 3 phenols, 3 fatty acids, 1 terpenoid) as shown *Table 3*. The fruit extracts of *C. fistula* contained several key classes of bioactive compounds, including phenols, flavonoids, alkaloids, terpenoids, and glycosides. Additionally, other classes of compounds, such as amino acids, fatty acids, and lignans, also partly contributed to the medicinal value of fruit extracts of *C. fistula (Table 4)*.

Effects of *C. fistula* Extracts Against the Adults of *H. contortus*

The adulticidal evaluation of various *C. fistula* extracts, compared with Levamisole and PBS, was demonstrated in adult motility assay *(Fig. 1, Fig. 2, Fig 3, Fig. 4)*. *Fig. 1* and *Fig. 2* illustrate the time-dependent paralysis and mortality of *H. contortus* exposed to different concentrations of fruit and root extracts of *C. fistula*, respectively. While *Figures 3* and *4* are presenting the corresponding effects of stemand leaves extracts of *C. fistula* in comparison with Levamisole and PBS controls. At the highest concentration (50 mg/mL), the fruit ethanol extract and leaves ethyl acetate extract demonstrated the most pronounced effects, causing complete paralysis and mortality of adult *H. contortus* (10.00±0.00) within 4 and 6 h, respectively. Levamisole caused complete paralysis of all adult worms (10.00±0.00) within 2-4 h of exposure, whereas no immotile worms (0.00±0.00a) were observed in PBS during the 12-h assay. These effects were statistically comparable to Levamisole ($P>0.05$), whereas lower concentrations and other extracts showed significantly reduced activity ($P<0.05$) compared to the standard drug. Overall, the results indicate that specific extracts, particularly fruit ethanol, exhibit potent adulticidal activity with efficacy approaching that of the reference anthelmintic.

The anthelmintic efficacy of the tested *C. fistula* extracts

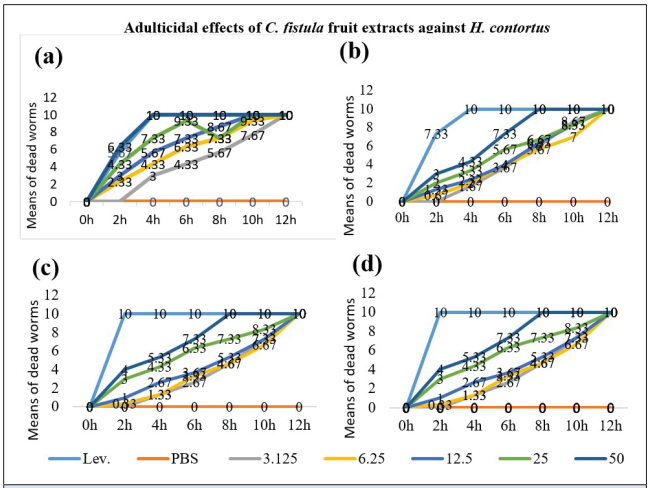

Fig 1. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* fruit extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: **(a)** ethanol fruits' extract, **(b)** methanol fruits' extract, **(c)** ethyl acetate fruits' extract, **(d)** aqueous-methanol fruits' extract. The values are means of immotile/dead parasites ± SEM: standard error of mean. The figure is exhibiting the means of dead worms vs the tested time duration of 12 h

Table 2. *Phytochemical compounds detected in LC-MS study in different C. fistula stem extracts*

Phyto-Constituents		**Class Name**	**RT (min.)**	**% Area**	**MW (g/mol)**	**% Abund.**
Bioactive metabolites present in stem ethanol extract	Adipic acid	Fatty Acids	1.81	3.70	146.1	6.84
	Beta Asarone	*Phenyl propanoids*	4.91	2.80	208.3	3.73
	Erucamide	Fatty Acids	5.51	4.01	337.6	2.93
	Luteolin	Flavonoids	5.41	2.71	286.3	2.01
	Quercetin	Flavonoids	5.81	4.42	302.3	1.70
	Parinaric Acid	Fatty acids	6.80	10.9	276.5	2.71
	22E-Stigmasta-5,22-Di-en-3-Ol	Terpenoids	5.51	6.05	412.7	1.17
	Lupa-12,20(29)-Di-en-3-One	Terpenoids	3.99	7.65	422.8	2.90
	4-Methoxy, Cinnamic Acid	Phenolic Acids	2.72	7.80	178.2	1.72
	Kaempferol	Flavonoids	1.41	12.6	286.3	1.65
	n-Hexadecanoic Acid	Fatty acids	1.87	3.98	256.4	4.57
	Barbaloin	Phenols	1.41	3.44	418.5	1.27
	Trimethylglycine	Amino Acids	2.21	3.61	117.2	1.74
	Quinine	Alkaloids	6.80	12.5	324.4	6.23
Bioactive metabolites present in stem methanol extract	Aloe Emodin	Anthraquinone	3.01	10.75	270.3	2.02
	4-Methoxy Cinnamic Acid	Phenolic Acids	1.01	2.12	178.2	2.21
	3-Hydroxy-3,7-Dimethyl-6- Octenedioic Acid	Fatty Acids	2.90	6.81	186.3	2.52
	Quercetin	Flavonoids	1.31	1.51	302.3	1.11
	Lup-20,29-en-28-al, 3β-Hydroxy	Terpenoids	1.78	6.55	641	1.23
	Erucamide	Fatty Acids	6.13	1.36	337.3	3.94
	Betulin	Terpenoids	7.13	3.22	442.7	6.77
	Kaempferol	Flavonoids	4.30	1.16	286.2	1.79
	Nervonic Acid	Fatty Acids	1.58	2.87	366.6	5.28
	Palmitic Acid	Fatty Acids	1.79	4.05	256.4	5.09
	Abietic Acid	Terpenoids	1.02	1.11	302.5	1.13
	Apigenin	Flavonoids	3.23	6.04	270.3	1.06
Bioactive metabolites present in stem' ethyl acetate extract	α,ω-Dicarboxylic Acid	Fatty acids	2.83	1.05	286.4	4.62
	Epicatechin	Flavonoids	1.07	1.83	290.2	1.11
	4-Hydroxycoumarin	Phenols	4.83	2.38	162.1	1.35
	8-Hydroxyquinoline	Phenols	2.49	3.07	145.2	2.24
	Quinine	Alkaloids	1.30	3.01	324.4	6.75
	Caffeic Acid	Phenols	3.02	1.61	180.2	2.01
	Barbaloin	Phenols	1.40	2.95	418.4	1.04
	Valine	Amino Acids	3.41	1.41	117.1	1.41
	Aloe Emodin	Fatty Acids	1.25	1.31	270.2	7.34
	Betuline	Terpenoids	1.31	1.31	442.7	1.81
	Rhamnetin	Flavonoids	1.33	2.40	316.2	1.36
	Oleanolic acid	Terpenoids	8.61	1.21	456.7	5.92
	Lupa-12,20(29)-Di-en-3-One	Terpenoids	1.11	1.10	422.7	3.21

Table 2. *Continue*

Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in stem aqueous methanol extract	α,ω-Dicarboxylic Acid	Fatty Acids	1.87	6.77	146.2	2.18
	(-)-Epigallocatechin	Flavonoids	1.64	6.74	458.4	6.01
	3-pyridinol	Phenols	1.12	5.19	95.1	1.02
	Quinine	Alkaloids	2.88	3.77	324.4	1.20
	Valine	Amino Acids	1.77	1.43	117.2	1.31
	Callosobruchusic Acid	Fatty Acids	1.91	2.82	200	2.03
	Aloe Emodin	Fatty Acids	1.07	3.08	270.2	6.68
	Oleanolic Acid	Terpenoids	4.39	8.35	456.7	3.41
	Ferulic Acid	Phenols	1.51	4.45	194.2	1.11
	7-ethoxycoumarin	Phenols	2.71	1.11	190.3	1.20
	Apigenin	Flavonoids	1.53	1.62	270.2	2.01
	3,6,2-Trimethoxyflavone	Flavonoids	243	1.81	313.1	1.98

RT: Retention time, MW: Molecular weight, abund.: abundance

against adult *H. contortus* followed the order: fruits' ethanol extract (4 h) > leaves' ethyl acetate extract (6 h) > fruits' ethyl acetate and methanol extracts = roots' ethanol extract (8 h) > fruits' aqueous methanol extract = leaves' ethanol extract = roots' methanol, ethyl acetate and aqueous methanol extracts (10 h) > stems' methanol, ethanol and ethyl acetate extracts = leaves' methanol extract (12 h). Overall, the fruit extracts demonstrated greater potency than the roots, stem, and leaf extracts. The activity of all extracts increased significantly in a time- and dose-dependent manner ($P<0.05$), with the highest concentration (50 mg/mL) showing the strongest effect. Moreover, no inhibition of motility (0.00±0.00a) was detected at 3.125 mg/mL after 2 h of contact.

Effects of *C. fistula* Extracts Against the Eggs of *H. contortus*

In the egg hatch assay, various *C. fistula* extracts inhibited hatching of *H. contortus* eggs significantly compared with PBS and Oxfendazole *(Fig. 5, Fig. 6)*. *Fig. 5* depict the inhibitory effects of fruit and root extracts *(Fig. 5-a,b)* on egg hatching at different concentrations, respectively. While *Fig. 6* show the corresponding effects of leaves and stem extracts *(Fig. 6-a,b)* in comparison with Oxfendazole and PBS controls. A clear dose-dependent inhibition of egg hatching was observed across all extracts. At the highest concentration (50 mg/mL), the ethanol fruit

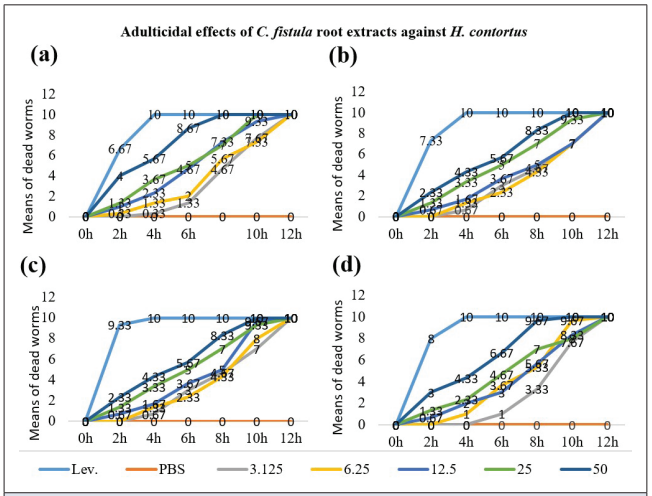

Fig 2. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* root extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: **(a)** ethanol roots' extract, **(b)** methanol roots' extract, **(c)** ethyl acetate roots' extract, **(d)** aqueous-methanol roots' extract. The values are means of immotile/dead parasites ± SEM: standard error of mean. Figure is exhibiting means of dead worms vs tested time duration of 12 h

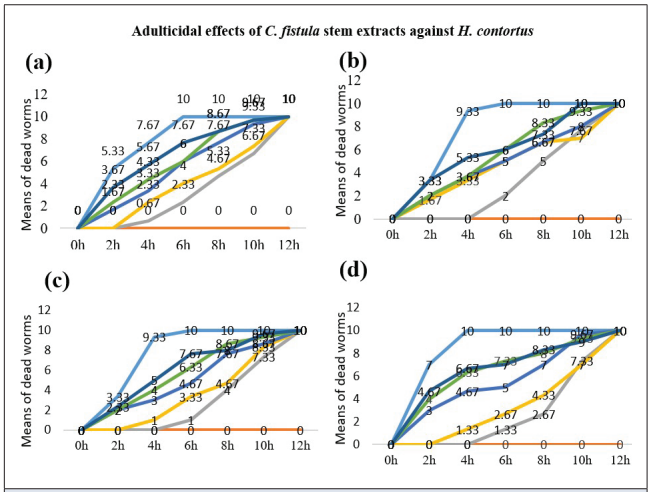

Fig 3. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* stem extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: **(a)** ethanol stem' extract, **(b)** methanol stem' extract, **(c)** ethyl acetate stem' extract, **(d)** aqueous-methanol stem' extract. The values are means of immotile/dead parasites ± SEM: standard error of mean. Figure is exhibiting means of dead worms vs tested time duration of 12 h

Table 3. *Phytochemical compounds detected in LC-MS study in different C. fistula roots' extracts*

Phyto-Constituents		**Class Name**	**RT (min.)**	**% Area**	**MW (g/mol)**	**% Abund.**
Bioactive metabolites present in roots' ethanol extract	Mahuannin-D	Phenols	3.81	1.91	528.5	2.29
	Vitispirane	Terpenoids	2.36	2.48	192.2	2.96
	Catechin Hydrate	Flavonoids	1.94	1.64	290.3	2.48
	Lansioside A	Terpenoids	4.11	11.11	659.9	17.3
	Resveratrol	Stilbenoids	2.82	3.85	228.2	2.59
	4O-Methyl melleolide	Sesquiterpenoids	4.66	3.98	414.5	1.76
	Levulinic Acid	Fatty Acids	2.69	1.65	116	2.53
	Protecatechuic Acid	Phenolic Acids	2.66	3.71	154	2.15
	Traumatin	Fatty Acids	5.16	21.17	212.3	21.50
	Gallic Acid	Phenolic Acids	1.71	2.06	170.2	1.54
	Tyramine	Amino Acids	2.08	1.42	137.2	1.56
	p-Coumaric Acid	Phenolic Acids	1.51	1.61	164	2.68
	Catechin	Flavonoids	7.96	5.26	290.3	3.23
	Phytol	Terpenoids	6.51	4.22	296.5	3.67
Bioactive metabolites present in roots' methanol extract	Isoquercitrin	Flavonoids	3.57	1.95	464.5	1.84
	Isoacteoside	Glycosides	1.87	2.15	664.6	1.74
	Gallic Acid	Phenolic Acids	1.54	3.65	170.1	3.11
	Demethoxy curcumin	Polyphenols	1.67	3.55	368.4	3.21
	3,4,5,7,8-Pentamethoxy Flavanone	Flavonoids	3.82	2.33	372.4	2.68
	6β-D-Glucopyranosyl-4,5-Dihydroxy-3,7-Dimethoxy Flavone	Flavonoids Glycosides	1.33	4.43	4773.2	8.66
	Caffeic Acid	Polyphenols	1.91	5.48	180.2	8.95
	Pilosanol A	Flavonoids	6.61	8.03	540.6	8.32
	Tyramine	Amino Acids	2.15	14.6	137.3	7.21
	6-OE-Caffeoylquinic Acid	Terpenoid	1.79	2.87	376.4	3.18
	Senampeline-A	Alkaloids	1.48	1.70	473.5	2.23
	Armillarivin	Terpenoids	2.87	3.18	384.5	2.14
	Fluprednisolone	Steroids	3.38	1.45	378.4	1.50
Bioactive metabolites present in roots' ethyl acetate extract	Rosmarinic Acid	Phenolic Acids	3.78	6.27	360.3	7.54
	Salicylic Acid	Phenolic Acids	2.24	1.97	138.1	1.68
	3R-Hydroxy-β-ionone	Sesquiterpenoids	1.76	7.70	208.3	4.03
	Quercetin,3O-Rhamnoside-7-O-Glucoside	Glycosides	3.08	3.15	610.5	2.17
	Protecatechuic Acid	Phenolic Acids	1.51	8.47	154.1	6.72
	Armillarivin	Terpenoids	1.72	2.28	384.5	3.11
	Epicatechin	Flavonoids	2.11	8.55	290.2	5.03
	B-sitosterol	Phytosterols	1.82	3.77	414.7	4.30
	Luteolin	Flavonoids	3.49	9.76	286.2	8.62
	Catechin	Flavonoids	1.81	1.84	290.3	1.51
	Myricetin	Flavonoids	1.54	2.25	318.2	2.92
	Gallic Acid	Phenolic Acids	2.73	3.86	170.1	5.26
	Silymarin	Flavonoids	1.94	3.55	482.4	4.32

Table 3. *Continue*

Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in roots' aqueous methanol extract	Pantothenic Acid	Vitamins	1.31	4.82	219.2	8.38
	Silidianin	Flavonoids	1.28	1.34	482.4	1.40
	Astragalin	Flavonoids	1.69	2.72	448.4	1.57
	Catechin	Flavonoids	1.26	4.42	290.3	4.05
	Rutin	Flavonoids	1.83	2.01	610.5	2.57
	6β-D-Glucopyranosyl-4,5-Dihydroxy-3,7-Dimethoxy Flavone	Flavonoids Glycosides	2.61	1.68	4773.2	2.75
	2,6-Di-O-Acetylononin	Phenols	2.54	7.65	514.5	2.77
	Myricetin	Flavonoids	1.44	1.51	318.2	1.97
	Vanillylmandelic Acid	Phenols	1.74	1.45	166.1	2.33
	Gallic Acid	Phenolic Acids	2.86	1.33	170.1	1.39
	Perillic Acid	Terpenoids	2.28	5.66	166.2	3.70
	Dodecanedioic Acid	Fatty Acids	1.83	2.14	200.3	3.15
	4O-Methyl melleolide	Sesquiterpenoids	1.65	3.44	414.5	5.85

RT: Retention time. MW: Molecular weight, abund.: abundance

extract exhibited the strongest inhibitory effect (90.33%), whereas the methanolic root extract showed the lowest activity (56.17%). Statistical analysis revealed that the inhibitory effects of the extracts were significantly higher than the negative control (PBS) ($P<0.05$). Moreover, the activity of the most effective extracts, particularly the fruit ethanol extract, was comparable to that of the standard drug Oxfendazole ($P>0.05$), while less active extracts showed significantly lower efficacy ($P<0.05$). Overall, fruit extracts demonstrated the highest ovicidal activity, followed by stem, leaf, and root extracts.

The highly potent fruit ethanol extract inhibited 68.33% of eggs hatching even at the lowest tested dose (3.125 mg/mL). At the highest concentration (50 mg/mL), the anthelmintic activity of the various *C. fistula* extracts against *H. contortus* eggs was recorded as follows: ethanol fruits' extract (90.33%), methanol stem' extract

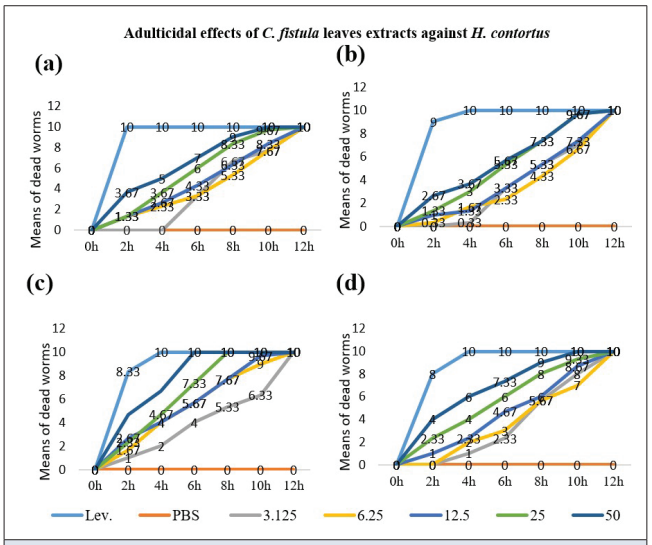

Fig 4. Photomicrographs illustrate the *in vitro* adulticidal activity of various *C. fistula* leaf extracts compared with Levamisole and PBS on the motility of *H. contortus*. Anthelmintic efficacy is shown for: **(a)** ethanol leaves' extract, **(b)** methanol leaves' extract, **(c)** ethyl acetate leaves' extract, **(d)** aqueous-methanol leaves' extract. The values are means of immotile/dead parasites ± SEM: standard error of mean. Figure is exhibiting means of dead worms vs tested time duration of 12 h

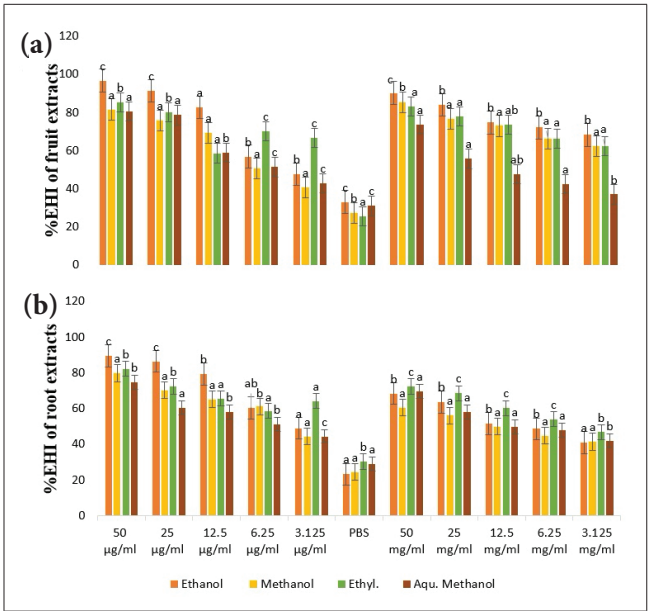

Fig 5. The percentage inhibition of egg hatching against *H. contortus* eggs by **(a)** *C. fistula* fruit extracts and **(b)** *C. fistula* root extracts is presented in comparison with various doses of Oxfendazole. Distinct letters (a, b, c) within each column indicate statistically significant differences among the treatment groups. Figure is showing Oxfendazole concentrations in µg/ml and plant extract doses in mg/ml. Ethanol: Ethanol fruit/root extract. Methanol: Methanol fruit/root extract. Ethyl.: Ethyl acetate fruit/root extract. Aqu. Methanol: Aqueous methanol fruit/root extract

Table 4. *Phytochemical compounds detected in LC-MS study in different C. fistula fruits' extracts*

Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in fruits' methanol extract	1,1,3-TriethoxyPropane	Esters	2.69	1.18	176.3	5.63
	Caprylic Acid	Fatty Acids	4.16	0.55	144.2	8.71
	6-Chloro-4-Phenyl-2-Propylquinoline	Alkaloids	19.9	23.75	256.7	38.6
	Methyl Salicylate	Esters	5.20	1.44	152.1	4.92
	Decanoic Acid	Fatty Acids	6.74	0.74	200	6.23
	8-Methylnonanoic Acid	Fatty Acids	9.82	5.28	171.2	9.14
	Pentacosane	Alkanes	17.2	0.92	352.7	9.83
	Griseophenone Trimethyl Ether	Benzophenones	17.6	0.02	387.2	5.41
	Pancracine,2O-Dimethyl-	Alkaloids	17.6	0.02	287.3	39.9
	3-Hydroxy-4'-methoxy-6-methylflavone	Flavonoids	18.8	3.95	282	56.62
	4Epi-DehydroAbietinol Acetate	Terpenoids	19.5	0.85	328.5	42.6
	Dioctyl Terephthalate	Esters	18.85	5.71	376.5	46.74
	2-Ethyl-Oxybenzylidene Acetophenone	Flavonoids	19.4	7.29	252.3	78.7
	Antioxidant 425	Phenols	18.1	1.78	340.5	53.42
	N-Heptafluorobutyryl-2-Aminofluorene	Aryl Amines	17.89	0.61	181.2	37.73
	6-Chloro-2,4-Diphenyl Quinoline	Alkaloids	17.89	0.62	315.8	31.54
Bioactive metabolites present in the ethanol extract of fruits	Gallic Acid	Phenolic Acids	8.73	9.82	170.1	27.74
	Beta-Sitosterol Acetate	Terpenoids	14.54	15.31	456.3	31.32
	Trifolin	Flavonoids	18.63	7.84	448.1	21.53
	2,2-DiethoxyPropane	Acetons	17.74	16.82	132.20	20.81
	Corchorifatty Acid F	Fatty Acids	17.52	21.61	328.2	13.32
	n-Hexadecanoic Acid	Fatty Acids	15.64	7.82	256.4	25.53
	4-Epi-DehydroAbietinol Acetate	Terpenoids	19.53	0.85	328.5	34.75
	Stigmasterol	Terpenoids	12.51	9.81	412.37	26.72
	p-Coumaric Acid	Phenolic Acids	13.72	11.52	164	19.95
	Myricetin-3O-Galactoside	Flavonoids	23.511	21.6	480.09	26.73
	6-Chloro-4-Phenyl-2-Propylquinoline	Alkaloids	19.99	23.75	256.6	14.44
	3,4-O-Dimethyl Gallic Acid	Benzoic acids	32.32	25.5	198.05	23.72
	1-Caffeoyl-beta-D-Glucose	Phenolic Acids	11.71	13.5	342.09	17.1
	Di-hydroquercetinGlucosyl Rhamnosy	Flavonoids	29.93	27.3	613.11	22.74
Bioactive metabolites present in the aqueous methanol extract of fruits	3O-Caffeoyl-4O-Sinapoylquinic Acid	Phenolic Acids	9.72	11.74	560.3	13.78
	Dodecanedioic Acid	Fatty Acyls	7.88	16.72	230.2	15.57
	Sinapinic Acid	Phenolic Acids	3.68	3.21	224.2	3.71
	Gallic Acid	Phenolic Acids	7.92	8.74	170.2	7.89
	Isoquercetin	Flavonoids	6.44	7.87	464.2	9.87
	Corchorifatty Acid, F	Fatty Acyls	12	20	328.2	9.72
	Decanoic Acid	Fatty Acids	6.75	0.75	200.1	7.98
	1,1,3-TriethoxyPropane	Esters	2.70	1.19	176.4	4.31
	p_Coumaric Acid	Phenolic Acids	17.7	13.7	164.1	9.83
	4-Propylphenol	*Alkylbenzene*	8.75	1.73	136.1	7.34
	Syringic Acid	Phenolic Acids	1.09	3.55	194.4	7.71
	Caprylic Acid	Fatty Acids	4.17	0.56	144.2	3.71
	Pyridoxine	Vitamins	18.99	19.38	169.2	21.71
	N-Acetyl-L-Phenylalanine	Aromatic	13.80	14.18	207.2	13.78
	Ethyl Palmitate	Fatty Acids	23.46	24.53	284.5	26.76

Table 4. *Continue*

Phyto-Constituents		Class Name	RT (min.)	% Area	MW (g/mol)	% Abund.
Bioactive metabolites present in fruits' ethyl acetate extract	Prenylated Chalcone	Flavonoids	21.2	23.72	354.4	27.7
	Pentacosane	Alkanes	17.2	0.93	352.7	5.68
	1,1,3-TriethoxyPropane	Esters	20	20.4	182.1	17.2
	2,2-dimethylphenyl 2-chloroacetate	Esters	9.83	5.29	198.3	7.90
	Perillic acid	Terpenoids	7.84	9.82	166.1	10.7
	Isocapric acid	Fatty Acids	9.83	5.29	171.3	8.77
	Gallic acid	Phenolic Acids	5.07	3.19	170.2	9.82
	Octadecane	Alkanes	17.2	7.93	254.5	13.5
	16-Hydroxypalmitic acid	Fatty Acyls	13.7	15.7	272.2	3.72
	Griseophenone, trimethyl ether	Benzophenones	17.6	9.03	387.2	13.1
	n-Propyl acetate	Esters	17.7	13.8	102.2	9.09
	Myricetin-3-Galactoside	Flavonoids	5.07	7.14	480.1	9.09
	Taxifolin 3-O-rhamnoside	Flavonoids	11.1	13.1	613.1	9.20
	Amyrone succinyl	Terpenoids	13.7	9.18	524.5	10.1
	Astragalin	Flavonoids	1.74	3.46	448.1	5.68
	Melilotoside	Aromatics	13.7	13.7	326.1	15.6
	p_coumaric acid	Phenolic Acids	2.09	3.19	164.1	9.20

RT: Retention time. MW: Molecular weight, abund.: abundance

(85.83%), methanol fruits' extract (85.33%), ethyl acetate leaves' extract (83.83%), methanol leaves' extract (83.50%), ethyl acetate fruits' extract (83.33%), aqueous-methanol stem' extract (81%), ethanol leaves' extract (79.17%), aqueous-methanol fruits' extract (73.67%), aqueous-methanol roots' extract (69.67%), ethanol stem' extract (78.5%), ethyl acetate stem' extract (77.33%), ethyl acetate roots' extract (63.67%), aqueous-methanol leaves' extract (76.17%), and methanol roots' extract (56.17%).

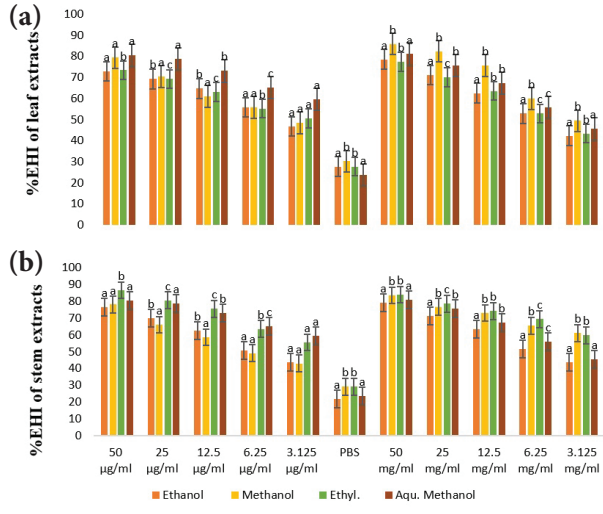

Fig 6. The percentage inhibition of egg hatching against *H. contortus* eggs by **(a)** *C. fistula* leaf extracts and **(b)** *C. fistula* stem extracts is presented in comparison with various doses of Oxfendazole. Distinct letters (a, b, c) within each column indicate statistically significant differences among the treatment groups. Figure is showing Oxfendazole concentrations in µg/ml and plant extract doses in mg/ml. Ethanol: Ethanol leaf/stem extract. Methanol: Methanol leaf/stem extract. Ethyl.: Ethyl acetate leaf/stem extract. Aqu. Methanol: Aqueous methanol leaf/stem extract

Effect of Ethanol Fruit Extracts and Crude Fruit Powder on Parasite Load in Goats

The *in vivo* anthelmintic activity of *C. fistula* fruit ethanol extract demonstrated the substantial fecal egg count reduction (*Table 5*). These results illustrate that *C. fistula* holds significant potential for managing gastrointestinal nematode infections in small ruminants. At the dose of 1000 mg/kg b.wt., the goats received ethanol fruit extracts of *C. fistula,* which exhibited the highest efficacy percentage of 90.78% (95% CI), while the goats treated with fruit powder of *C. fistula* exhibited parasites eggs reduction of 70.20%. A single dose oral administration of albendazole (10 mg/kg b.wt.) resulted in an 87.91% reduction in parasite load in the treated goats. Moreover, the efficacy of the ethanol fruit extract revealed an outstanding improvement from the 7th to the 21st day post-treatment. Post-treatment of animals with the fruit ethanol extract of *C. fistula* revealed the highest efficacy in comparison to crude fruit powder and albendazole. There was recorded a significant difference ($P<0.05$) between animals receiving the ethanol extract and plant powder of *C. fistula* in comparison with the control groups.

Table 5. *The in vivo anthelmintic efficiency of fruit powder and fruit ethanol extract of C. fistula against gastrointestinal nematodes in experimentally infected goats as evaluated by faecal egg count reduction assay*

Groups	Treatments	% EPG ± SEM			
		Day 0	Day 7	Day 14	Day 21
T0	No treatment	9.46±4.09^{a}	8.89±3.89^{a}	5.39±3.61^{a}	3.38±4.07^{a}
T1	ABL 10 mg/kg	43.51±2.17^{d}	67.61±1.81^{e}	78.29±1.71^{f}	87.91±2.08^{g}
T2	CFE 250 mg/kg b.wt.	9.40±3.08^{a}	18.75±3.67^{b}	45.62±3.71^{c}	74.58±1.58^{e}
T3	CFE 500 mg/kg b.wt.	8.19±2.57^{a}	23.99±3.09^{bc}	62.93±2.21^{d}	79.27±1.57^{f}
T4	CFE 1000 mg/kg b.wt.	7.15±3.61^{a}	21.57±3.29^{b}	59.80±2.07^{d}	90.78±0.55^{g}
T5	CFP 250 mg/kg b.wt.	8.29±3.89^{a}	11.11±1.46^{a}	33.17±1.47^{b}	52.54±1.78^{c}
T6	CFP 500 mg/kg b.wt.	11.37±3.60^{a}	24.61±3.09^{bc}	40.30±1.51^{c}	69.53±1.57^{d}
T7	CFP 1000 mg/kg b.wt.	17.19±3.08^{b}	34.63±1.47^{d}	51.21±1.57^{cd}	70.20±2.51^{d}

Values are means of % EPG ± SEM: Eggs per gram ± Standard error of mean. ABL: Albendazole. Day-0 (pre-treatment % EPG). Day 7, 14, 21 (post-treatment % EPG). P<0.05. CFE: C. fistula fruit ethanol extract. CFP: C. fistula fruit powder

Discussion

The differences observed in extract yield among the various plant parts may be attributed to their distinct biochemical compositions, as certain bioactive compounds exhibit higher solubility in specific solvents than in others [11]. Variations in extract yield are largely influenced by the polarity of the extraction solvents, and differences in biological activity likely reflect variations in the concentration of bioactive constituents being extracted [25]. In this study, the fruit methanol extract (13%) and fruit ethanol extract (12.5%) of *C. fistula* produced the highest yields. The value of *in vitro* assays becomes particularly evident when assessing the effects of plant extracts on multiple developmental stages of parasites: eggs, larvae, and adults, using techniques such as the egg hatch inhibition assays, larval motility inhibition assay, larval exsheathment inhibition test, and adult motility assay [26]. Such multi-stage evaluations help reduce the potential for parasite resistance [27].

In the initial *in viro* screening, emphasis was on inhibiting egg hatching, especially in helminths exhibiting resistance to benzimidazoles [28]. The egg hatch assay is widely used as a standard approach for assessing the anthelmintic potential of plant-derived compounds [29]. Active phytochemicals can penetrate the eggshell and cause paralysis of L1 larvae. This mode of action not only decreases the parasite's load in the host but also lowers the pasture contamination with infective eggs [30]. The effect of protein fractions from *Combretum leprosum* and *C. fistula* was studied against the larvae and eggs of nematodes infecting goats by Silva et al.[31]. *C. leprosum* exhibited limited inhibition of egg hatching and larval development. *C. fistula* exhibited strong suppressive effects on both stages in naturally infected animals. The egg's inhibitory activity observed in the present study is consistent with the findings reported by Silva et al.[31].

Plant-derived extracts can impair the immune defenses of parasites and induce paralysis by damaging the worm's cuticle [32]. In present study, the strongest anthelmintic activity was observed in the fruit ethanol extract and the leaves ethyl acetate extract at 50 mg/mL, which induced complete paralysis and mortality of adult *H. contortus* (10.00±0.00), respectively within 4 and 6 hours of exposure, demonstrating activity comparable to the synthetic drug Levamisole. The anthelmintic effects of *C. fistula* against the adult worms of *H. contortus* have not yet been studied. However, the adult motility effects of *C. fistula* in current research work present a close correlation with the anthelmintic effects of *C. alata*; belong to the same family as *C. fistula* [33]. *C. alata* exhibited significant ovicidal and vermicidal effects against the eggs and adults of *H. contortus* upon 6 h exposure to its freeze-dried hydro-acetonic and aqueous extracts [33].

Furthermore, parasite egg counting is essential for determining the *in vivo* efficacy of plant extracts. Increasing the dosage of plant extracts is often justified by the possibility that parasites may develop resistance to the administered compounds, necessitating higher concentrations to achieve the desired therapeutic effect [24]. Plant-derived treatments are generally considered effective when they produce more than a 90% reduction in both adult parasites and eggs per gram (EPG) [34]. According to Reinecke [35], a fecal egg count reduction (FECR) above 90% denotes high efficacy, 80% indicates moderate efficacy, and 60% reflects low efficacy; any FECR below 60% is deemed ineffective. In the present study, the fruit ethanol extract of *C. fistula* at 1000 mg/kg body weight produced the highest FECR of 90.78% (95% CI) indicating high anthelmintic efficacy and representing the most potent treatment among the tested extracts.

In the present study, LC-MS analysis revealed the presence of various bioactive including alkaloids, flavonoids,

amino acids, phenols, alkanes, terpenoids, esters, sterols, fatty acids, and glycosides. *C. fistula* was found to be a rich source of flavonoids, alkaloids, and phenols, and its analysis confirmed the presence of important anti-parasitic constituents, such as quercetin, kaempferol, gallic acid, and hydroxyl-cinnamic acid derivatives (including *p*-coumaric, ferulic, and chlorogenic acids). There is substantial scientific evidence supporting the anthelmintic potential of these pharmaceutical constituents [36]. The presence of key phenolic constituents, such as ferulic acid, gallic acid, vanillic acid, quercetin, and kaempferol, in *Artemisia brevifolia* and the anthelmintic effects of these phyto-components against gastrointestinal nematodes in sheep [37]. The results of the present research show agreement with Hussain et al.[37] and Abdullah et al.[38]. Abdullah et al.[38] studied that the ethanol leaf extract of *C. fistula* contained the highest levels of flavonoids and alkaloids. The presence of these bioactive compounds across different *C. fistula* extracts in the current study indicated their potential as an effective alternative to synthetic drugs for the treatment of haemonchosis and other parasitic diseases.

In conclusion, the anthelmintic activity of various parts of *C. fistula* against *H. contortus* and other gastrointestinal nematodes has been clearly demonstrated. This species belongs to the family *Fabaceae,* contains several anti-parasitic active pharmaceutical ingredients, such as gallic acid, quercetin, kaempferol, and hydroxycinnamic acid derivatives (including *p*-coumaric, ferulic, and chlorogenic acids), which have also been identified and isolated from other members of the same family. Thus, the fruits of *C. fistula* appear to be a rich source of secondary metabolites with significant anthelmintic activity, offering their potential as a promising natural alternative to conventional synthetic drugs for controlling gastrointestinal parasitic infections. Future studies will incorporate coproculture and larval identification to better establish species-specific efficacy and to further align the *in vitro* and *in vivo* components of the work.

Declarations

Availability of Data and Materials: The data sets generated during the current study, including experimental data from plant extracts and laboratory animals, are available from the corresponding authors (AA & KM) on reasonable request.

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Ethical Approval: Animals experiments were conducted in strict adherence to the Guidelines for the Welfare and Ethical Review of Laboratory Animals and were conducted following approval from the Animal Ethics' Committee of the Islamia University of Bahawalpur (Approval # 456.ORIC)

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