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Therapeutic anthelmintic efficacy of *Calotropis procera* and *Acacia nilotica* against gastrointestinal nematodes of Mouflon sheep

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ABSTRACT

Small ruminants are especially susceptible to gastrointestinal nematodes when kept in captivity. Medicinal plants have bioactive compounds and are extensively used for treatment helminthes infection. This study's objective was to evaluate the possible anthelmintic activity of in-vitro leaf extracts from *Calotropis procera* (*C. procera*) and *Acacia nilotica* (*A. nilotica*) in three different solvents: aqueous, methanolic and ethanolic. The two assays used for the evaluation of anthelmintic activity against gastrointestinal nematodes of Mouflon sheep were egg hatch inhibition (EHI) and larval development inhibition (LDI). For EHI, aqueous, methanol and ethanol extracts of *C. procera* and *A. nilotica* had LC_{50}=0.28, 0.23 and 0.17 mg/ml and 0.42, 0.36 and 0.27mg/ml, respectively while aqueous, methanol and ethanol extracts of *C. procera* and *A. nilotica* had 0.38, 0.26 and 0.19 mg/ml and 0.32, 0.25 and 0.17mg/ml, respectively. The most effective was ethanol extract for both egg hatching inhibition and larval development assay. These results showed that the leaves of *C. procera* and *A. nilotica* possess ovicidal and larvicidal properties against nematodes of wild sheep.

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Introduction

Helminthes are parasitic worms that feed on a living host to gain nourishment and safety, while inflicting terrible nutrient absorption, weakness and other disorders within the host. The foremost gastrointestinal nematodes infecting and affecting small ruminants are *Haemonchus contortus, Teladorsagia Circumcincta* and *Trichostrongylus spp* (Magrone, 2014). Nematodiosis is a serious illness that affects newborn lambs in the spring and early summer. Young lambs might endure unexpected deaths and epidemics of diarrhoea when grazing on pastures infested with high numbers of larvae, which grow from eggs left by lambs during past grazing seasons. Infestations of parasitic gastro-enteritis commonly result in excessive diarrhoea, impaired performance, weight loss, emaciation, and in rare instances anaemia as well.

In addition, parasitism may have indirect outcomes on metabolism inclusive of mobilisation of proteins for an immune-response, decreased feed intake because of anorexia or extended susceptibility to different pathogens (Chauvin et al. 2010). According to studies, sheep GI parasites have a significant impact on public health and are implicated in transmission of zoonotic diseases to humans, either directly through contact with sheep faeces or indirectly through consumption of contaminated food or water (Jackson et al. 2009). One of the major health problems substantially restricting animal productivity is helminthes parasitism, more specifically gastrointestinal parasitism (Veerakumari, 2003).

Sheep are even-toed ungulates of the order Artiodactyla, which includes ruminants. Sheep are mostly related to the wild mouflons that once

roamed Asia and Europe. Sheep were among the first animals to be domesticated for use in agriculture, and they are raised for milk and meat (lamb, hogget, or mutton).

Mouflon sheep (*Ovis orientalis*) entitles an animal species that is widely hunted and produced in Central Europe. In addition to the protected population of the 700 Urial, the value of the trophy of the Punjab Mouflon sheep (*O. orientalis*) is also under pressure from hunting due to trophy value, and in some parts of Pakistan. It has declined sharply in a short period of time and is persevered only as a low-density species. Mouflon sheep are very sensitive to many parasitic diseases (Muelleriosis, Trichostrongylosis, Fasciolosis, Nematodiosis or Dicrocoeliosis etc). These diseases cause health problems and animals die prematurely and cause economic losses to breeders. Therefore, anthelmintic drugs are often used in the breeds of Mouflon sheep (*O. orientalis*), especially in game parks and farms.

Parasites cause many problems for wildlife, and although wildlife often seems that they have adapted to the presence of parasites, they do not adapt to the adverse effects of parasites (Ese, 2005). In the wild, animals have a certain ordinary resistance or balance to the parasite, but because of changes in environmental and living conditions, animal behavior and ecology is affected hence the possibility parasitism in captive animals is increased (Atanaskova, 2011). Through their hands, clothes, shoes, food, and work instruments, the crew has a significant impact on the spread of parasites among the animals in zoos. Animals themselves, when they are moved from one enclosure to another without receiving sufficient parasite treatment, are another potential source of parasite transmission.

Chemotherapy is a chief remedy used for helminthes infection in animals. However, because of increasing improvement of anthelmintic fight and the restrained availability of industrial drugs as well as the excessive cost of such synthetic medicines developing countries to take a look at the anthelmintic plants for treatment of different diseases historically (Mali and Mehta, 2008; Kaplan, 2004). To combat bovine and human gastro-intestinal parasites, a sizable number of plant-based products are utilized. Phytotherapeutic pills are safe, non-toxic, biodegradable, and leave no traces in products used with animals. The World Health Organisation (WHO, 2018) anticipated that 80% of the population of growing nations depend upon traditional medicine in general plant capsules for health care needs. Plants are richest supply of bioactive natural chemical compounds on this planet. The consequences recommend that the Phytochemical drugs for using diverse illnesses (Ganga et al. 2007).

The traditional medicinal herb known as *Calotropic procera* is frequently used to cure a variety of illnesses. It is an upright, perennial plant that flourishes luxuriantly in wasteland. On Earth, bioactive organic chemical molecules are most abundant in plants. They serve as the main repository for secondary metabolites such flavonoids, alkaloids, terpenoids, steroids, and many others. Various plant extracts or bioactive compounds are used in traditional medicine. The results support the idea that the stem, leaves, and flower's phytochemical qualities can be used to treat a variety of diseases (Shrivastava et al. 2013; Tiwari et al. 2014). In semi-arid areas, young *C. procera* pods, leaves, and flowers are utilized as cattle feed. Numerous phytochemicals found in plants have a wide range of pharmaceutical and traditional medical uses. Two related Asclepiadaceae species, *C. procera* and *C. gigantean*, are widely used to treat a variety of diseases and physiological issues. Both species have significant disease-curing abilities against a variety of infectious agents, including microorganisms, viruses, fungi, protozoans, and worms. The active ingredients in *C. procera* have shown properties including cytostatic, cytotoxic, wound healing, antipyretic, procogulant, analgesic, anticonvulsant, antidiabetic, hepatoprotective, antitumor, anti-coccidial, antiarthritic, anticancer, anti-fertility and anti-inflammatory properties.

Acacia nilotica is likewise a popular ornamental street tree in India and Pakistan (Malviyal et al. 2011). Farmers in several underdeveloped nations use the leaves and legumes of the *A. nilotica* species to feed tiny ruminants. A. nilotica, often referred to as "Desi Kikar" locally, has been recommended for its anthelmintic qualities (Bachaya et al. 2009). The fact that the tannin content of *A. nilotica* has been studied for potential anthelmintic qualities has also increased interest in the plant (Athanasiadou et al. 2000).

It has an exciting array of medical benefits with ability anti-oxidant. This plant provides a number of corporations among which might be alkaloids, phenols, volatile crucial oils and resins, phenolic glycosides, steroids, oleosins, terpenes and tannins. Different components of this plant include the roots, leaves, bark, seeds, gum, flowers, and immature pods act as anti-most cancers, antimutagenic, antimutagenic, vasoconstrictor, spasmogenic, anti-asthamatic, anti-pyretic, anti-plasmodial cytotoxic, anti-platelet agregatory, anti-diabetic, molluscicidal, anti-fungal, anti-plasmodial, inhibitory interest towards Hepatitis C virus (HCV) and human immunodeficiency virus (HIV)-I and antioxidant sports, anti-hypertensive, anti-bacterial and anti-spasmodic sports, and are also engaged for the handling of various illnesses within the indigenous system of drugs. This analysis focuses on the specific phytochemical makeup, therapeutic applications, and pharmacological properties of distinct components of this multiuse.

Due to the emergence of anthelmintic resistance, the scarcity of commercial drugs for rural populations, the high price of such manufactured medicines, and the increasing development of anthelmintic resistance, there is rising interest in the ethno-veterinary method to study the anthelmintic properties of plants that are traditionally utilised by local farmers in various parts of the world (Mali and Mehta, 2008; Kaplan, 2004).

This study's goal aimed to assess the *in-vitro* anthelmintic efficacy of aqueous, methanol, and ethanol extracts of both plants *C. procera* and *A. nilotica*.

Materials and Methods

Phytochemical screening of plant extracts

Plant extracts were screened for phytochemical content using standardised techniques (Bagewadi et al. 2012). A standard protocol was followed to perform a qualitative analysis on the aqueous, methanolic, and ethanolic extracts of *A. nilotica* and *C. procera* (Ait) leaves for the existence of plant phytoconstituents such as flavonoids, saponins, alkaloids and tannins (Kokate et al. 1999; Khandelwal., 2000)

Collection of samples

A total of 160 fecal samples of Mouflon sheep (*Ovis orientalis orientalis*) of all ages and sexes were collected at the Wildlife Park Gatwala, Faisalabad Pakistan from August 2018 to March 2019, immediately after defecation. Ten samples were collected fortnightly. When a faecal sample was taken from the ground, proper precautions were taken to avoid contamination. Faeces weighing between 60 and 100 grammes were put in a plastic bag. Every sample was stored in its own polythene bag. To the lab, Department of Zoology, Government College University, Faisalabad, the faecal samples were brought in polythene bags with the relevant labels, numbers, and information. Incorporating the particular helminth-specific modification of the McMaster (1998) method, samples of 5 g of faeces were weighed and well mixed in 40.5 ml of saturated salt (NaCl) solution, which has a specific gravity of about 1.20 (Cringoli et al. 2004). Then, the material was strained through a sieve with an approximately 1-mm-wide opening, and the waste was discarded. After that, 0.5 ml of the resulting solute was pipetted onto the second chamber of a double-chambered McMaster slide after being mixed one more time. The slide was then permitted to sink for 5 minutes to allow all faeces with a specific gravity greater than 1.20 to sink and helminth eggs (particularly nematodes, which can be recovered using a saturated salt flotation solution; Taylor et al. 2007; Cringoli et al. 2004; MAFF, 1986); to float to within a visible microscopic range. The chambers were examined under 10X magnification using a compound microscope. All eggs were observed in the two separate chambers (both inside and outside the designated grid) to calculate the Faecal Egg Count (FEC).

Egg hatch assay (EHA)

The World Association for the Advancement of Veterinary Parasitology's (WAAVP) guidelines were followed (Cole et al. 1992). *C. procera* and *A. nilotica* extracts in aqueous, methanol, and ethanol were used as the test treatments. Ivermectin (99.8% pure standard reference) was employed as a positive control and untreated eggs in water served as the negative control. 200 eggs and 1.5 mL of water were present in each test tube. In a total volume of 3 ml, plant extracts in aqueous, methanol, and ethanol at concentrations of 3.0, 1.5, 0.75, 0.37, and 0.18 mg/ml, respectively, were made together with water containing eggs. Ivermectin was dissolved and diluted in dimethyl sulfoxide (DMSO) at concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.0156 g/ml. The test tubes were then covered and kept in an incubator at 27°C for 48 hours. The test was repeated three times for each concentration. Unhatched eggs and hatching larvae were counted at 40X magnification using a dissecting microscope.

Larval development test

First-stage larvae were produced after 24 hours of egg incubation at 27°C. 30 ml plastic cups were used for the experiment, which was modified somewhat from the Costa et al. (2008) approach. In a nutshell, 6g of sheep faeces free of gastrointestinal nematodes, 6 successive concentrations of plant extracts, an aliquot of 1 ml containing 100–200 first-stage nematode larvae, water containing larvae, and a volume of egg free of faeces were combined. Ivermectin was dissolved in distilled water at six different doses as a positive control, and eggs in parasite-free faeces were used as a negative control. All samples underwent a 7-day incubation period at room temperature. Each cup's wall was thoroughly cleaned with 12 cc of water at the end of the seventh day in order to gather the larvae. Then, all L3-stage larvae were computed using a microscope at 40X magnification and one drop of Lugol's iodine solution.

Statistical analysis

By using probit analysis in comparison to the logarithm of extract concentration, egg hatch assays (EHA) were transformed. SPSS version 13.0 was used to do the statistical analysis. From linear regression, the extract concentration needed to inhibit hatching at the 50% lethal concentration (LC50) was determined.

Results

Phytochemical screening of plant extracts
Plant extracts were screened for phytochemical content using standardised techniques (Bagewadi et al. 2012). The extracts of experimental plants showed different types of organic compounds i.e. alkaloids, saponin, flavonoids and tannin revealed from the specific reagents.

Eggs hatching inhibition
The result of extracts of two medicinal plants in aqueous, methanol and ethanol at different concentrations on egg hatching inhibition of nematodes of wild sheep was 0.1875-3mg/ml. Results of an assay to determine how well two plants' solvent extracts hinder egg hatching shown in Table 1. Egg hatching inhibition increased in a dose-dependent manner (P 0.05) with increasing extract concentrations. The greatest concentration of nematodes that prevented egg hatching (98.8%) in the ethanol solution was 3 mg/ml. Inhibition of nematode egg hatching by leafy extracts of *C. procera* and *A. nilotica* in water, methanol, and ethanol was (93.9%), 94.7%, and 98.8%, respectively. 100% of egg hatching inhibition was triggered by ivermectin at a dosage of 0.150 mg/ml. The suppression of egg hatching is not affected by the nematode control group. *C. procera* and *A. nilotica's* LC50 values in aqueous, methanol, and ethanol extracts were 0.28, 0.23, and 0.17 mg/ml, respectively. For *C. procera*, the values were 0.42, 0.36 and 0.27 mg/ml.

Larval development inhibition
Two medicinal plants' leaf extracts demonstrated prevention of larval growth against nematodes in wild sheep in aqueous, methanol, and ethanol solvents. The leafy extract of *C. procera* in ethanol solvent at a dosage of 3 mg/ml showed a maximum 99.8% suppression of larval growth, whereas the leaf extract of *A. nilotica* showed a 96.8% inhibition. Similarly, the aqueous, methanol and ethanol extracts at different concentration were also displayed larval development inhibition compared with Ivermectin the positive control (P<0.05) (Table 2). The LC_{50} of *C. procera* in aqueous, methanol and ethanol extracts were 0.38, 0.26 and 0.19 mg/ml and *A. nilotica* 0.32, 0.25 and 0.17mg/ml respectively. It was detected that the ethanol extract of *C. procera was* the most effective against larval development of parasites (P<0.05).

Discussion
The nematicidal activity of leaves of two plants extracts (in aqueous, methanol and ethanol) at different concentrations was observed for larval development and inhibition of eggs *in vitro*. The development of nematode larvae and the hatching of nematode eggs were more consistently influenced by the ethanol foliar extracts of *C. procera* and *A. nilotica*. Alkaloids, flavonoids, and tannins are known to be present in large quantities in these plants. Previous research suggested that plant extracts' saponin mixtures have larvicidal and ovicidal efficacy against parasitic worms (Doligalskaa et al. 2011; Marie-Magdeleine et al. 2009, 2010; Eguale et al. 2007; Eguale et al. 2011). Additionally, both directly and indirectly, tannin compounds have significant effects on gastrointestinal nematodes (Athanasiadou et al. 2001; Hoste et al. 2006; Iqbal et al. 2007; Marie-Magdeleine et al. 2009). Numerous Acacia species, including *A. cyanophylla* (1), *A. karoo* (34), *A. nilotica* (34, 35), *A. pannutula* (2,3) and *A. polycantha* (42), as well as the bark of *A. mangium* (54) and *A. mearrsii (*69), have been reported to contain anthelmintic properties. An anthelmintic effect of *A. nilotica h*as been documented (Eguale et al. 2006; Bachaya et al. 2009). Major phytochemicals in Acacia spp. are tannins and flavonoids (Malan and Roux, 1975; Tindale and Roux, 1969; Devi and Prasad, 1991), free amino acids (Evans and Bell, 1979), cyanogenic glucosides (Secor et al. 1976), labdane diterpenes (Forster et al. 1985), acacipetalin (Seigler et al. 1978) and proanthocyanidins and other phenolic compounds (Dube, 1993). The effects of the substances or chemical groups mentioned above, individually or collectively, may be responsible for *A. nilotica's* effectiveness as an anthelmintic.
The results of prior studies on other helminth parasites and the anthelmintic activity of plant extracts at different concentrations of *C. procera* reported in the current investigation are in agreement (Ranjit et al. 2012; Roy et al. 2010; Ghangale et al. 2009; Tariq et al. 2008). The LC_{50} of *C. procera* ethanolic and aqueous extracts differed depending on the solvent used to extract the most powerful active components, despite having similar efficacy. The range of secondary metabolites present may be responsible for the anthelmintic effects of the aqueous, methanolic, and ethanolic extracts of *C. procera* and *A. indica*. Plant extracts passed a preliminary phytochemical screening that revealed the presence of alkaloids, tannins, phenols, flavonoids, and saponins, whose intensities varied between plants and with the extract solvent. Additionally, it was clear that *C. procera* was more effective than *A. nilotica*, an activity that could be linked to the many alkaloids and phenols present in the substance. Saponins in the crude extracts of *C. procera* and *A. nilotica* may have caused the parasites to refuse food, starve, and eventually die from a lack of energy. There is evidence that tannins play a part in the management of helminthes (Kotze et al. 2009; Forbey et al. 2009). Numerous *in-vitro* investigations have reported on the nematicidal effects of tannin extracts and provided evidence of the anthelmintic activities of condensed tannins (Ademola and Idowu 2006; Molan et al. 2003). It's probable that the effects of the two plants' aqueous, methanol, and ethanol extracts were similar due to

the tannins present. According to the results of the current in vitro study, ethanolic extracts of both *C. procera* and *A. nilotica* showed significant

Table 1: Eggs hatching inhibition percentage +SD and LC_{50} of the egg hatching assay for the different plants extracts, compared to negative and positive control

Plant name and concentration (mg/ml)	Hatching inhibition (%) ± SD		
	Aqueous	Methanol	Ethanol
C. procera			
3mg/ml	93.9±5.34	94.7±3.45	98.8±6.67
1.5mg/ml	85.6±7.28	76.3±4.36	88.4±7.78
0.75mg/ml	63.5±5.47	58.2±7.23	64.4±9.24
0.375mg/ml	42.7±3.83	36.3±11.25	48.7±3.97
0.1875mg/ml	17.6±6.08	23.5±8.20	32.5±8.86
$LC_{50\ mg/ml}$	0.28	0.23	0.17
A. nilotica			
3mg/ml	83.3±3.46	88.5±7.86	95.8±6.75
1.5mg/ml	73.7±2.74	66.9±7.71	88.6±7.32
0.75mg/ml	55.2±11.2	58.2±3.76	63.5±5.34
0.375mg/ml	34.6±9.26	38.5±9.10	45.3±5.35
0.1875mg/ml	15.2±6.05	23.5±4.46	24.3±6.52
$LC_{50\ mg/ml}$	0.42	0.36	0. 27
Albendazole	100.00±0.00	100.00±0.00	100.00±0.00
(-ve control)	0.00±0.00	0.00±0.00	0.00±0.00

Table 2: Development of larval inhibition percentage +SD and LC_{50} of the larval development assay for the different plants extracts, compared to negative and positive control

Plant name and concentration (mg/ml)	Hatching inhibition (%)±SD		
	Aqueous	Meth	Ethanol
C. procera			
3mg/ml	93.9±2.34	91.7±	99.8±3.16
1.5mg/ml	82.3±5.72	73.3±	68.4±7.64
0.75mg/ml	71.5±6.34	64.2±	52.8±4.73
0.375mg/ml	55.7±7.83	43.4±	31.3±5.36
0.1875mg/ml	23.9±3.06	19.5±	13.5±1.38
$LC_{50\ mg/ml}$	0.38	0. 26	0.19
A. nilotica			
3mg/ml	88.9±3.46	83.5±	96.8±3.25
1.5mg/ml	77.9±7.45	69.9±	68.6±11.32
0.75mg/ml	59.6±31.8	58.2±	54.5±4.65
0.375mg/ml	42.6±8.37	36.5±	24.3±2.94
0.1875mg/ml	21.2±3.65	18.5±	12.3±1.96
$LC_{50\ mg/ml}$	0.32	0. 25	0. 18
Albendazole	100.00±0.00	100.0	100.00±0.0
(Negative control)	0.00±0.00	0.00±	0.00±0.00

anthelmintic activity *in-vitro* against sheep nematodes and may be used as a replacement anthelmintic agent to treat gastrointestinal helminths in wild sheep and other ruminants.

The two herbs used in our investigation were successful in treating the intestinal worms that plagued wild sheep. Using this strategy in a traditional setting might be beneficial. To take into account the metabolism of extracts in the digestive system of ruminants kept in captivity. However, it would be necessary to carry out *in-vivo* parasitological studies.

This would likely be connected to the various chemical components recovered using various solvents, as well as the origin of parasites and prior contact with the plants. Other researchers (Suteky and Dwatmadji 2011; Costa et al. 2008) noticed a similar variance in potency and efficacy when they utilised different solvents for active ingredient extraction and noticed variable bioactivity outcomes.

Plant metabolites may function in an additive, synergistic, or antagonistic manner, operating at a single or numerous target locations, according to Wynn and Fougere (2007) and Briskin (2000) assessments. Therefore, it is probable that a variety of substances may have helped to the anthelmintic activity found in the two plant extracts. It's possible that the tannins in both the ethanol and the aqueous extracts from the two plants had identical outcomes. The two plants *C. procera* and *A. nilotica* were employed in this work to treat gastrointestinal nematodes in vitro, but in vivo parasitological studies would be required to take into account the metabolism of extracts in the gastrointestinal tract of wild sheep.

Conclusion

It was concluded from the present study that the most effective was ethanol extract for both egg hatching inhibition and larval development assay. The leaves of *C. procera* and *A. nilotica* possess ovicidal and larvicidal properties against nematodes of wild sheep.

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