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Anthelmintic activity of *Ocimum sanctum* leaf extract against ovine gastrointestinal nematodes in India

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ABSTRACT

Leaves of *Ocimum sanctum* have been traditionally used for various ethno-veterinary practices as well as medicinal purpose. *In vitro* ovicidal and larvicidal potential of crude aqueous and hydro-alcoholic extracts of the bulb of *O. sanctum* was investigated. Alkaloids, carbohydrates, steroids and tannins were identified in phytochemical analyses. The various blood parameters coupled marker enzymes and antioxidant status were also evaluated during *in vivo* trial. Aqueous extract showed better EC_{50} and EC_{99} values in comparison with methanolic extract in egg hatch assay and larval development test, respectively. However, in the larval paralysis test, both aqueous and methanolic extracts showed almost similar efficacy. A 77.64% reduction in fecal egg output was observed on day 14. No deleterious ill effect was found in any of the hematological and biochemical parameters suggesting that the plant could be safer for use in sheep.

1. Introduction

With the emergence of anthelmintic resistance, the parasitic control program suffered gradual setback in several countries forcing researchers to search for alternative control methods (Dhar et al., 1982). Anthelmintic resistance has been reported for almost all the commercially available anthelmintics in the Indian market (Jaiswal et al., 2013). Ethnoveterinary alternatives are considered to be of immense potential in overcoming anthelmintic resistance due to synthetic chemical compounds (Lans et al., 2007). Worldwide research has shown that certain plants can effectively be used to reduce the degree of parasitism and hence, they can be seen as promising alternatives to the conventional chemical anthelmintics (Githiori et al., 2006).

Ocimum sanctum (commonly known as Tulsi) is native to the Eastern World tropics and is widespread as a cultivated crop throughout India. The leaves are the most common medicinally used plant part worldwide for many centuries. In herbal medicine, it is used to prevent cancer (Pandey, 2009), used as an immunomodulator (Mukherjee et al., 2005), hepatoprotectant and in hematinic activities (Chattopadhyay et al., 1992). Besides these, it also possess some anthelmintic activity against roundworms particularly ascarids (Singh and Nagaich, 2000). The present study was designed to assess the anthelmintic potential of various extracts of leaves of *O. sanctum* on naturally occurring gastrointestinal nematodes of sheep using standard *in vitro* and *in vivo* tests.

2. Materials and methods

2.1. Collection and processing of plant material

Fresh leaves of *O. sanctum* were collected from the plants grown within the premises of Veterinary University, Mathura, India. The plants were later identified by the Department of Botany, Babu Shivnath Agrawal College, Mathura and the specimens were recorded in the form of voucher number (DUVASU-2014-OS) for further references. The leaves were cleaned from adulterants and air dried under shade at a well-ventilated place. The plant material was pulverized to powder form with a mixer grinder and stored in airtight containers.

2.2. Preparation of crude extracts

Extracts from the leaves of *O. sanctum* were prepared in water and methanol (S.D Fine Chem. Ltd.). For preparing the aqueous extract, 100 g of powdered leaves was taken in a beaker and suspended in 600 ml of water and was continuously stirred using magnetic stirrer for 4 hours. Again, for preparing the methanolic extract 100 g of powder was soaked in 500 ml of methanol solvent to exhaustion (120 h). The extraction was carried out in a percolator

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by a combination of maceration and percolation at room temperature. The filtrates of both extracts were collected through Whatmann filter paper No. 4. The removal of the solvent was performed at a temperature below 40 °C, under reduced pressure and a rotation speed of 20 rpm in vacuum rotary evaporator. The extract was scraped off, transferred to an air-tight container and stored in a freezer at –20 °C till subsequent use.

2.3. Determination of extraction yield (% yield)

The yield (%, w/w) from all the dried extracts was calculated as:

$$\text{Yield}\,(\%) = (W_1 \times 100)/W_2$$

where W_1 is the weight of the extract obtained after evaporation of the solvent; and W_2 is the weight of the plant powder. Later both extracts (100 mg each) were dissolved in 1 ml of distilled water to get 100 mg/ml concentration and subsequent lower dilutions were prepared (100–0.781 mg/ml) by using twofold dilution. The extracts were kept in airtight containers at 4 °C to avoid loss of any volatile principles or/and activities till further use.

2.4. Phytochemical analysis of crude extracts

Both extracts were tested for the presence of active principle like carbohydrate, steroids, tannins, flavonoids, alkaloids, glycoside, fat and proteins using standard procedures (Debela, 2002).

2.5. In vitro *tests*

2.5.1. Egg hatch test

The eggs of GI nematodes were recovered using the method described by Taylor et al. (2002) and recommended by the World Association for the Advancement of Veterinary Parasitology, WAAVP (Coles et al., 1992). Briefly, about 10 g of feces was collected directly from the rectum of naturally infected sheep and was mixed and homogenized with tap water. The mixture was sieved through a strainer and centrifuged for 2 minutes at 2000 rpm, the supernatant was discharged and after the tube was filled with saturated solution, coverslips were put over the meniscus for 5 minutes and were later washed to collect the eggs. The egg suspension, with a concentration of 100 eggs/0.2 ml, was distributed in a 24-multiwell plate (50 µl per well) along with 50 µl of extract of each tested concentration. Three replicates were made for each concentration. The plates were incubated, under humidified conditions, at 27 °C for 48 h and later a drop of Lugol's iodine solution was added to each well to stop further hatching. Hatched larvae and unhatched eggs were then counted under the microscope.

2.5.2. Larval development test

The Micro agar larval development test was performed as per the standard protocol of Coles et al. (2006). Briefly, 10 µl of individual concentration of each extract was placed in wells in triplicates and 150 µl of 2% bacto agar at 45 °C was added in triplicate form into each well and allowed to cool at room temperature. Thereafter, 10 µl of egg suspension, diluted in Amphoteracin B (1:1) was poured upon the cooled agar. Then, 10 µl of yeast extract, prepared in Earle's salt solution (Hubert and Kerboeuf, 1984), was added to it. The plates were sealed with parafilm and incubated at 25 °C for 7 days and the number of live L_3 in each well was counted thereafter.

2.5.3. Larval paralysis test

Larvae were cultured according to MAFF (1986) using Bearmann's apparatus. 100 µl of suspension (approx 100 larvae) was added to 100 µl of each concentration, in triplicate form, in the 96-well plate and was kept at room temperature for 24 h. The live (motile) and dead larvae were counted.

Albendazole at a dose of 0.125 mg/ml was used as a positive control while distilled water served as negative control in all the three tests (Kanojiya et al., 2015).

2.6. In vivo *test*

2.6.1. Fecal egg count reduction test (FECRT)

The same sheep flock that was used for the collection of eggs for the *in vitro* trial was again used for *in vivo* trial. The flock consisted of local Chokla breed of sheep that were naturally infected with gastrointestinal parasites. Single pooled larval culture, one from each group, was done on day 0 in order to find out the species of GI parasites involved. The flock was divided into three groups of fifteen animals each. Group 1 served as a negative control and received no treatment while Group 2 served as a positive control and was given a single dose of Albendazole at a dose of 7.5 mg/kg bodyweight. Group 3 was drenched a single oral dose of aqueous extract at a dose of 5 g/animal by making final volume of 5 ml with water (Kanojiya et al., 2015). Fecal samples of animals were individually collected on days 0, 7, 14 and 21 post-treatment. These samples were processed for egg per gram (epg) calculation using the McMaster method (Soulsby, 1982). FECRT efficacy was calculated according to Dash et al. (1988) using the following formula:

$$\text{FECR} = 100 \times \left(1 - \left[\frac{T2}{T1}\right]\left[\frac{C1}{C2}\right]\right),$$

where T is the average fecal egg count of treated group; C is the average fecal egg count of control group; 1 is the pre-treatment average fecal egg count; 2 is the post-treatment average fecal egg count.

Each group of sheep was housed separately but was allowed to graze altogether freely during the daytime. Again on day 21 post treatment, single pooled larval culture, one from each group, was done to identify the species of GI parasites surviving the herbal treatment.

2.7. Blood sample for hematology

Blood samples were obtained on day 0 and day 14 from each animal *via* Jugular vein puncture into 10 ml vacutainer. Different blood parameters were analyzed using Hematology analyzer (Diatron) and Semi auto chemistry analyzer (Electronics India) using serum [Serum Glutamic Pyruvate Transaminase (SGPT), Serum Glutami cOxaloacetic Transaminase (SGOT)], whole blood [Red Blood Cells (RBCs), White Blood Cells (WBCs), Hemoglobin (Hb), Hemocrit (HCT), Mean Corpuscular Volume (MCV), Red Blood Cell Distribution Width (RDWc), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelet Count (PLT), Mean Pack Volume (MPV), Procalcitonin (PCT), Lymphocyte %, Monocyte % and Granulocyte %] and plasma (Ferric Reducing Antioxidant Power FRAP). Total antioxidant activity was measured by FRAP assay (Benzie and Strain, 1999). Briefly, plasma (100 µl) was mixed with 3 ml of working FRAP reagent and absorbance was measured at 0 minute after vortexing. Thereafter, samples were placed at 37 °C in a water bath and absorbance was again measured after 4 min. Ascorbic acid standards (100 µM–1000 µM) were processed in the same way.

$$\text{FRAP value of sample}\,(\mu\text{mol/l}) = \frac{A-B}{X-Y} \times 100$$