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Amaranthus tricolor Linn. Leaf Extracts' In-vitro Anthelmintic Potential.

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ABSTRACT

Using acetone, ethyl acetate, and ethanol as solvents, the goal of the current research was to examine the anthelmintic potential of the Amaranthus tricolor Linn plant, which belongs to the Amaranthaceae family. The phytochemical components of the extracts were tested, and their vermicidal efficacy against adult Eisenia fetida earthworms was assessed. Comparatively speaking, phytochemicals were present in all of the extracts. The majority of the phytochemicals were present in the acetone extract, but the ethanol extract had less of them. In the bioassay, different concentrations (10–30 mg/mL) of each extract were examined, and the earthworms' paralysis and eventual death were tracked. Normal saline was used as the control group and albendazole as the reference standard. Every extract showed greater potency than the reference medication and dose-dependent anthelmintic action in both the measures (paralysis and death). The most promising result was the acetone extract (30 mg/mL), which paralyzed worms in 5 minutes and killed them in 13 minutes. The result implies that the A. tricolor Linn plant's acetone extract could be helpful as an anthelmintic. The current research provides scientific proof for the traditional use of this leafy vegetable as a vermicide and indicates that the leaves of the A. tricolor Linn plant are a good source of active chemicals with anthelmintic action. According to the early phytochemical study, the remarkable vermicidal activity of acetone extract may be attributed to the substantial presence of glycosidic and phenolic compounds in it.

Introduction

The prevalence of gastrointestinal helminthiasis infection by parasites such as hookworms and tapeworms is one of the notable health hazards, affecting 1.5 billion people worldwide.[1,2] The worm infection causes serious health conditions of anemia, diarrhea, vomiting, loss of appetite, acidity, and under nourishment, and leading to serious morbidity by affecting a large population.[3] As per the World Health Organization and pharmacologists, only a few drugs such as albendazole, mebendazole, benzimidazoles, piperazine, diethylcarbamazine citrate, ivermectin, and levamisole are used in the treatment of helminthiasis in human being.[2,4] These synthetic drugs show undesirable side effects, often become resistant to parasites, and are non-affordable by many poor people.[5]The inadequate availability of effective allopathic medicinal drugs, their adverse side effects, and the increasing resistance of gastrointestinal parasites towards synthetic anthelmintics create a problem in treating and managing this disease. Considering the facts, it is the need of the hour to develop an effective and alternative strategy against gastrointestinal helminths.

Anthelmintics from natural medicinal plant sources can provide an efficient and eco-friendly alternative to commercially available drugs. Anthelmintic plants, also known as vermifuges or vermicides, are used traditionally to expel the parasitic worms from the body either by causing distress or demise to the worms. Also, it was found that the leaf extract of a variety of medicinal plants shows anthelmintic properties when compared to other parts of the medicinal plant.[6] The plants of the Amaranthaceae family, such as *Amaranthus tricolor L.*

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exhibits alexeteric and anthelmintic properties.[7] In Maharashtra, the *A. tricolor* plant is locally known as "Laal Maath", a highly consumed leafy vegetable. It is often used in folklore medicines worldwide.[8,9] Traditionally, *A. tricolor L.* plant is reported in ayurveda as astringent in menorrhagia, leucorrhea, dysentery, colitis, cough, throat infection, toothache, bronchitis, eczema, piles, diarrhea, gonorrhea, impotence, and also used externally to treat mouth ulcers.[8, 10] A literature survey revealed that *A. tricolor L.* has wide spectrum of pharmacological activities: antibacterial, anti-diabetic, anti-inflammatory, antihyperglycemic, antimicrobial, anti-nociceptive, antioxidant activity, antiproliferative, antipyretic, cholestrolemic, cyclooxygenase enzyme inhibition, hematological, hepatoprotective, hypoglycemic, hypolipidemic and gastroprotective activities.[11-13]

Considering the various biological activities, the anthelmintic activity of different leaf extracts of *A. tricolor Linn.* was carried out in the present study. As per the available reports this is the first study of the anthelmintic activity of *A. tricolor L.* plant. Though, few attempts have been made on other *Amaranthus* genus plants, which cannot establish this plant's anthelmintic properties. M. Baral *et al.*[14] have evaluated the anthelmintic property of only water extracts of *A. spinosus Linn* plant against *Pheritima posthuma* and *Tubifex tubifex* worms. George B *et al.*[15] have investigated anthelmintic property of the aqueous extract of leaves of *A. dubius* plant along with the *Basella alba* and *Cleome gynandra* plants. Whereas, the Kumar *et al.* [16] have studied the anthelmintic property of methanolic extract of *A. caudatus Linn* plant. Their [14 -16] study lacks the screening of anthelmintic activity of plants with different solvents of varying polarity, therefore it is difficult to assess the nature of phytochemicals causing said anthelmintic activity, scientifically. Literature survey prompted us to explore the anthelmintic activity of anthelmintic activity of *A. tricolor L.* plant leaf with solvents of varying polarity. We have chosen the commonly used solvents for the extract preparation, which widely differ in their nature and polarity (ethyl acetate < acetone < ethanol). Since different polarity solvents extract the different type of phytochemicals, may help in identifying the substance with anthelmintic property. Therefore, this study aimed to determine the *in-vitro* anthelmintic activity of acetone, ethyl acetate and ethanol extracts of leaf of *A. tricolor L.* plant.

Materials and Methods

Plant Material

Fresh *A. tricolor L.* leafy vegetable was collected from Pune City, Maharashtra, India, cleaned, and air-shade dried under room temperature. The dried samples were further milled into a fine powder using a mortar pestle and stored in air-tight containers. The plant was taxonomically identified and authenticated at the Botanical Survey of India, Pune, where voucher specimen was deposited. The authentication number allotted is BSI/WRC/IDEN.CER/2019/H3/19.

Chemicals

Albandazole drug (GlaxoSmithKline) and normal saline were purchased from authorized pharmaceuticals. Ethanol, acetone and ethyl acetate solvents used during the experimental protocol were of analytical grade.

Experimental Animal

Adult earthworm species (*Eisenia fetida*) were used to study anthelmintic activity. The earthworms were procured from a local nursery. Collected from clammy soil and washed with normal saline to remove all fecal matter. The worms were 8 to 10 cm long and 0.2 to 0.3 cm in width were used for all the experimental protocols.

Preparation of Plant Extracts

Three different extracts of plant were prepared by refluxing (for 18 hours) weighed quantity (50 mg) of powdered leaf material separately in a known volume (250 mL) of ethanol, acetone, and ethyl acetate solvents. Solvents were recovered under reduced pressure to obtain the crude extracts, and respective extracts were screened for anthelmintic activity.

Phytochemical Screening

All extracts were screened for the presence of preliminary phytochemicals following standard protocols[17] and results were presented in Table.1.

Anthelmintic Activity

The anthelmintic activity was performed following the methodology as stated by Mali *et al.*,[18] with slight moderation. For the bioassay adult earthworm species, *E. fetida* was used in view of the fact that they bear a physical and physiological resemblance with the gastrointestinal roundworm parasite of individuals.[19] The ready availability of earthworms, additionally, makes them suitable for the purpose of the initial *in-vitro* evaluation of anthelmintics.[20] Albendazole drug with normal saline solution was used as standard reference and

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normal saline served as a positive control. Ethyl acetate, acetone, and ethanol extracts of the plants, in normal saline solution were used for the assay. Pure solvents served as negative control and 1% solution of each solvent with saline were used as solvent control. The time taken for complete paralysis and death of earthworms were recorded. External stimuli were applied to ascertain the paralysis time. The time taken by the worm to become motionless was considered as paralysis time and lethal time was ascertained by the death of the motionless worm followed by fading of its body color. All the results were shown in Table 2 and expressed as a mean ± SD of three worms in each group.

Administration of Extract

The animals were divided into sixteen groups, each consisting of three worms of approximately equal size. The earthworms were released into a Petri dish containing 20 mL of test solutions.

For evaluation of anthelmintic activity, three groups received standard albendazole drug suspended in normal saline in varying concentrations of 10 to 30 mg/mL. The same concentration (10–30 mg/mL) of acetone extract, ethyl acetate extract and ethanol extract in three separate sets of each. Petri dish containing normal saline solution acts as a negative control. Further, three sets served as solvent control and were treated with 1% ethanol, 1% acetone, and 1% ethyl acetate solutions, respectively.

Statistical Analysis

All the results were expressed as mean ± S.D. of three animals in each group. The data were verified as statistically significant using one-way ANOVA at 5% significance level ($p < 0.05$, $n = 3$).

Results and Discussion

Phytochemical Screening

Preliminary phytochemical screening depicts that all the leaf extracts of *A. tricolor L.* were enriched in phytochemical (Table 1). The test for alkaloid, tannin, phenol, glycosides, starch and sugars were positive for all the extracts. The tests for all phytochemicals were found to be positive for acetone extract. For ethyl acetate extract the test for all phytochemicals were found to be positive except steroids. The tests for all phytochemicals were found to be positive for ethanol extract except proteins, flavonoids and saponins.

Anthelmintic Assay

The leaf extracts of *A. tricolor L.* exhibits significant anthelmintic activity in a dependent manner (Table. 2, Fig. 1). All extracts demonstrated superior anthelmintic properties when compared to the standard albendazole drug. The normal saline water was used as a control. No symptoms of paralysis and death of earthworms were observed in normal saline water. All extracts were able to show greater anthelmintic activity than the standard drug at all doses. The most promising activity exhibited by the acetone extract of the *A. tricolor L.* causing the death of worms in most efficient manner, followed by the ethyl acetate and ethanol extract (Fig.1).

The anthelmintic activity of acetone extract turns out to be exceptionally effective, causing paralysis at 19, 7 and 6 minutes and death at 41, 22 and 13 minutes at 10, 20, and 30 mg/mL concentrations, respectively. The ethanol extract of *A. tricolor L.* at 10, 20, and 30 mg/mL concentration shows paralysis at 69, 55, and 43 minutes and death at 141, 117, and 98 minutes, and ethyl acetate extract of it shows paralysis at 25, 19 and 11 minutes and death at 45, 138 and 20 minutes, post-exposure (Table 2). Literature survey suggests that the paralysis and death of earthworms caused by the 30 mg/mL concentration of acetone extract of *A. tricolor L.* is of maximum efficacy than any reported case from Amaranthacea family.[14-16] As reported by M. Baral *et al.*[14] the water extracts (50 mg/mL) of *A. spinosus Linn* plant causes the paralysis and death of *Pheritima posthuma* and *T. tubifex* worms of annelida family in 18 and 33 minutes, respectively. While the aqueous extract of leaves of *A. dubius*[15] is reported to causes the paralysis and death of *E. fetida* earthworms in 18 minutes and 28 minutes, respectively, at a very high concentration of 300 mg/mL. The methanolic extract of *A. caudatus Linn*[16] plant takes the shortest time for paralysis (5.75 minutes) and death (8.5 minutes) of earthworms at higher concentration of 100 mg/mL.

Table 1: Phytochemical screening of the different extracts of *A. tricolor L.*

Phytochemicals	*AE*	*EAE*	*EE*
Protein	+	++	-
Alkaloid	+	++	++
Flavonoid	++	+	-
Steroid	+	-	++
Saponin	+	+	-
Tannin	++	+	++
Phenol	++	+	+
Glycoside	++	+	+
Starch	+	++	++
Sugars	+	+	+

*[(++): Strong presence, (+): Presence, (_): Absence, EAE: Ethyl acetate extract, AE: Acetone extract, EE: Ethanol extract,]

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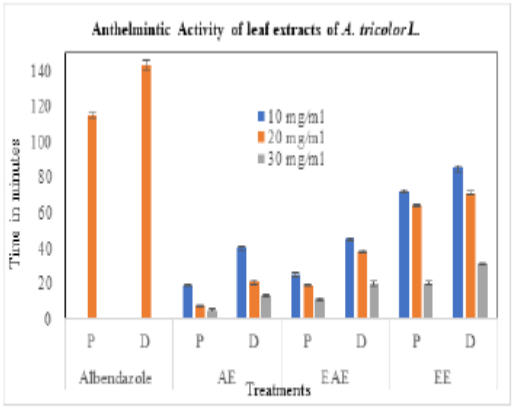

(P: Paralysis; D: Death; SD = Standard deviation; AE: Acetone extract; EAE: Ethyl acetate extract; EE: Ethanol extract)

Fig.1: Anthelmintic Activity of various extracts of *A. tricolor* L. plant leaves. Results are given as mean ± SD. Significance at $p < 0.05$.

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Table 2: Anthelmintic potency of *A. tricolor L.* leaf extracts

Treatment	*Concentration (mg/mL)*	*Paralysis Time (min.)*	*Death Time (min.)*
Albendazole	20	115 ± 1	143 ± 2.52
Acetone	10	19 ± 1	40 ± 1
	20	7 ± 0.58	21.3 ± 1.2
	30	5 ± 1.53	13.3 ± 0.6
Ethyl acetate	10	25 ± 2	45 ± 1
	20	19 ± 1	38.3 ± 0.58
	30	11 ± 1.53	20 ± 1
Ethanol	10	72 ± 2	85.3 ± 1.5
	20	64 ± 1	71 ± 1
	30	20 ± 1.53	31.3 ± 0.6
Control (Saline) (Positive control)	---	---	---
1% Solvents (Acetone/Ethyl acetate/ Ethanol) (Negative control)	---	---	---

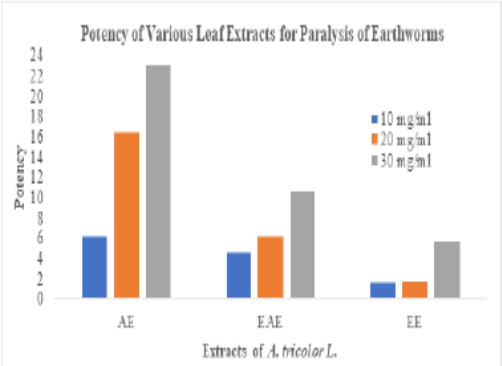
*--- no paralysis, no death;

**AE: Acetone extract; EAE: Ethyl acetate extract; EE: ethanol extract

Table 3: Potency of various extracts

Concentration (mg/mL)	AE		EAE		EE	
	P	D	P	D	P	D
10	6.1	3.5	4.6	3.2	1.6	1.7
20	16.4	6.5	6.1	3.8	1.8	2.0
30	23	11	10.5	7.2	5.8	4.6

*AE: Acetone extract; EAE: Ethyl acetate extract; EE: ethanol extract; P = Paralysis; D = Death



(P: Paralysis; D: Death; AE: Acetone extract; EAE: Ethyl acetate extract; EE: Ethanol extract)

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Fig. 2: Potency of various extracts of *A. tricolor* L. leaf for paralysis of earthworms

Further, the results indicates that the potency (for paralysis as well as death) of acetone extract is highest at all concentrations, followed by the ethyl acetate and ethanol extract. The potency (efficacy) of extracts was found to be inversely proportional to the time taken for paralysis and death of the worms. As shown in Table 3, the potency for various extracts is increasing with increasing the concentration of extracts and maximum potency for paralysis and death of earthworms is observed at 30 mg/mL.

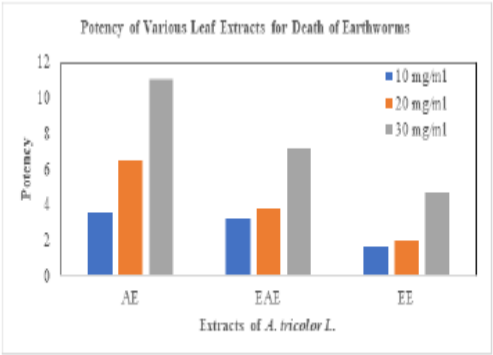
Paralysis of Earthworms at various Concentrations of Extracts

Acetone extract causes the paralysis of earthworms in minimum time followed by the ethyl acetate extract and then by ethanol extract (Fig. 2). For paralysis of worms the acetone extract (30 mg/mL) is five times more potent than the ethanol extract, and almost two times more potent than the ethyl acetate extract. For acetone extract, by increasing the concentration in double amounts (from 10–20 mg/mL), potency is significantly increased up to 2.7 times and efficacy of extract increases by 3.8 times when concentration becomes three-fold, from 10 to 30 mg/mL.

Death of Earthworms at various Concentrations of Extracts

Acetone extract causes the death of earthworms in minimum time followed by the ethyl acetate extract and then by ethanol extract (Fig. 3). For death of worms the acetone extract at a concentration of 30 mg/mL is approximately 2.4 times more potent than the ethanol extract, and almost 1.5 times more potent than the ethyl acetate extract. For acetone extract, by increasing the concentration in double amounts (from 10–20 mg/mL), potency is significantly increased up to 1.9 times. The extract's efficacy becomes three times when concentration is increased by three times, from 10 to 30 mg/mL.

The preliminary phytochemical screening of all crude extracts reveals the presence of alkaloid, tannin, phenols, glycosides, starch, flavonoids, and tannins as major constituents (Table 1). Literature survey reveals that the plants' secondary metabolites may interfere with parasites' metabolism processes by inhibiting the glucose uptake and affecting the worms' energy-generating mechanism.[21-24] The phyto-constituents like alkaloids, phenolics and tannins have significant anthelmintic activity.[21] The alkaloids are reported to cause the paralysis of the worms by attacking on their central nervous system.[22] Polyphenolic compounds are known for its parasiticidal activity towards worms possibly by increasing the host



(P: Paralysis; D: Death; AE: Acetone extract; EAE: Ethyl acetate extract; EE: Ethanol extract)

Fig. 3: Potency of various extracts of *A. tricolor* L. leaf for death of earthworms.

resistance.[23] Tannins have been reported to interfere with energy generation mechanism of worms by uncoupling the oxidative phosphorylation or by binding to the free protein of the gastrointestinal tract of the worms and leading to their death.[24] Albendazole-like worm expellers cause paralysis of the worms resulting in their expulsion by peristalsis. Primarily, albendazole increases the chloride ion conductance of worm muscle membrane, causing hyper polarization and excitability reduction, which that leads to muscle relaxation and flaccid paralysis of worms.[25] From the above study, it can be said that the secondary metabolites present in *A. tricolor L.*, may have produced similar effects, causing the death of the worms. Therefore, the claim of *A. tricolor L.* plant as a potent anthelmintic has been confirmed as the various extracts shown excellent activity against *E. fetida* earthworms. Further, the efficacy of an extract depends on its transit time in the gastrointestinal tract of humans. Being a leafy vegetable *A. tricolor L.* plant extracts may get highly absorbed in the body and have enough transit time to act. Therefore, the gradual increase in the extract dose exhibited a stepwise increase in the activity (Fig. 1). It is concluded from the above results that, leaves of *A. tricolor* L. plant has great potential as an anthelmintic agent, possessing varying dose-dependent activities for all extracts. The various leaf extracts exhibited the trend of activity as Acetone > Ethyl acetate > Ethanol. In the present study, the experimental evidence obtained in the laboratory model validates the traditional use of these leafy vegetables as vermicides. Further study is needed to isolate and identify the active compounds in the

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crude extracts and *in-vivo* trials may be conducted for use in livestock on a scientific basis.

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