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PHYTOCHEMICAL ANALYSIS & ANTHELMINTIC ACTIVITY OF LEAVES OF LEUCAENA LEUCOCEPHALA

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ABSTRACT

Helminthic infections are chronic illnesses both in human beings and cattles. *Pheritima postuma* earthworms are a known helminthic and they have anatomical and physiological similarities with intestinal roundworm parasite present inside human beings. Medicinal plants have been using as health care products from a long time ago that have been used by humans in the form of traditional medicines all over the world. The use of herbal drugs extract and their remedies have increased throughout the world against the increasing use of synthetic drug medicines. **Objective:** To study the phytochemical analysis and anthelmintic activity of various prepared extracts obtained from the leaves of Leucaena leucocephala against earth worms Pheritima postuma. **Material and Methods:** 3 concentrations (10 mg/ml, 25 mg/ml and 50 mg/ml) of several prepared extracts such as chloroform extract, methanol extract, ethyl acetate extract and Petroleum ether extract were tested in earth worms and results were expressed in the terms of time taken for paralysis and time taken for death of worms. Tinidazole (10 mg/ml) was taken as standard drug and tween 80 as a vehicle control group. **Result: A**ll the prepared extracts were tested at 10 mg/ml, 25 mg/ml and 50 mg/ml concentrations, in which petroleum ether extract shows much better anthelmintic activity than all other prepared extracts and than the reference standard drug Tinidazole. **Conclusion:** This study review has demonstrated that the petroleum ether extract of *Leucaena leucocephala* leaves possess significant *in vitro* anti-worm activity at the tested concentrations.

KEYWORDS: Leucocephala, helminths, anthelmintic, Subabul, petroleum ether, squalene.

INTRODUCTION

The word 'helminth' came from a Greek word "helmins" which means parasitic worm. Helminthic worms are highly predominant and depend on the other species. These can exist as individuals or as parasites dependent on plant or animal hosts. In human beings helminthic infections are known as one of the most common infections. It affects a large amount of total world population. In most of the developing nations they possess a huge threat to public health and take part in the occurrence of pneumonia, anaemia, eosinophilia and malnutrition. A proper study is needed for the effective control of helminths which includes different using methods of anthelmintic.[1-3]

Leucaena leucocephala plant is a member of the family fabaceae. It is commonly called as Subabul, White Popinac, White Lead tree, Jumbay and Wild Tamarind.[4-5] *L. leucocephala* is found in India in Himachal Pradesh and it is native to Southern Mexico and Northern Central America.[6] The word 'Leucaena' is derived from the Greek word 'leuc' and 'caen' which refers to whitish flowers. It is possesses highly nutritious value. This tree is mainly important due to its high medicinal value and it also produce firewood, timber, human food, green manure, shade and also to control erosion.[7-11] It is a perennial, evergreen, drought-tolerant shrub about 10 m in height.[12] It has whitish yellow flowers and having long oblate pods. Seeds are in brown colour with shining seed coat.[13-14] It contains various active chemical constituents such as squalene, oxalic acid, pentadecanoic acid, heptacosanoic acid, hexadecanoic acid, phytol, Octacosane, hexatriacontane, tetratetracontane, methyl ester and a lot more.[15] The main active chemical constituent of *L. leucocephala* is squalene (about 41.02%)[16-17] It contains various phytochemical properties such as antibacterial[18], antihistaminic, antioxidant[19-20], anticancer, anti-inflammatory, antidiabetic[21], antitumor, antimicrobial[22-24], anti-proliferative, hepatoprotective, anti-androgenic, hypocholesterolemic, diuretic, pesticide and nematicide.[15] This plant is known to be a worm repellent. The seeds of *L. leucocephala* contain various phytochemical properties and used to treat stomach ache, as contraception and abortifacient.[25-27]

MATERIAL AND METHODS

Collection and authentication of plant material

The plant material of *Leucaena leucocephala* inquired in the current study was collected from the local areas of the district Solan of Himachal Pradesh between the months of April and May, 2018. Plant material was cleaned properly, at first it washed with tap water and then

with pure or distilled water to remove all the dust particles or impurities and then kept in shade to dry properly. This plant species was identified and authenticated as *Leucaena leucocephala* by Dr Y.S. Parmar University of Horticulture & forestry, Nauni, Solan, Himachal Pardesh (H.P), vide Book no. 3818, Reciept no. 009 and reported as the sample is linked with UHF- Herbarium with field book No. 13578. The herbarium is kept at Nauni University for reference.

Collection of Worms

The Indian earthworms named *Pheritima postuma* were collected from the Agriculture office of Chambaghat, Solan district, (H.P).

Earth worms[28-32]

The Indian adult earthworms *Pheritima postuma* were collected from the humid soil and washed with normal saline to remove all adhering faecal or unwanted matter which were further used for the study of anthelminthic activity. The earthworms *P. postuma* were about 5-7 cm long and 0.2- 0.4 cm wide. These were used for all experimental procedures because it has anatomical and physiological similarities with intestinal roundworm parasite present inside human beings.

Chemicals and Reagents

The following drug and chemicals were used.

Drug: Tinidazole

Chemicals: ammonia solution, acetic anhydride, Dragendorff's reagent, Ether, Ethanol, Ethyl acetate, hydrochloric acid, Methanol, Molisch's reagent, Mayer's reagent, Million's reagent, Petroleum Chloroform sulphuric acid, Tween 80.

Preparation of the plant extracts

The plant material was properly cleaned, at first washed with the tap water and then with pure or distilled water to remove all impurities or dust particles and then kept in shade to dry properly. Powdered material was then extracted in Soxhlet apparatus, using Chloroform, Petroleum Ether, Ethyl acetate and methanol respectively. Then the extracts were concentrated to semi-solid masses and stored in an air sealed container in a refrigerator for further uses.

Phytochemical analysis of the prepared extracts[33]

The preliminary phytochemical analysis of the leaves of *l. leucocephala* plant extract mainly done for the evaluation of the various phytochemical constituents such as anthraquinones, alkaloids, flavonoids, proteins, sugars, sterols, tannins, Saponins and terpenoids were present in plant extracts prepared in several solvents such as Chloroform, Ethyl acetate, Petroleum Ether and methanol of *L. leucocephala*.

Test for Alkaloids (Mayer's test)

Mayer's Test: Took 2ml plant extract and 2ml concentrated HCL were added. Mayer's reagent was further added in a little amount. Green colour or white precipitate obtained which shows that the alkaloid groups are present.

Test for anthraquinones

1g of the plant extract was at first boiled with 20 ml of H_2SO_4 and filtered during heated state. The filtrate material was shaken-up with 10 ml chloroform. The layer of chloroform was pipetted into another test tube and 2 ml dilute ammonia (NH_4) was added. Now the prepared solution was kept and observed for changes in colour.

Test for flavonoids (Ferric chloride test)

Took approx. 1g of the plant extract and boiled in 10 ml pure or distilled water then filtered. 4 ml of filtrate was taken and few drops of 10% ferric chloride solution was added. Violet or Green-blue colour obtained which indicates the existence of a phenolic hydroxyl group.

Test for proteins (Xanthoproteic test)

Little amount of the plant extract was dissolved in 4 ml distilled water, 1 ml concentrated nitric acid (HNO_3) further added in the solution. Yellow colour obtained which shows the presence of proteins.

Test for sugars (Fehling's test for free reducing sugar)

Took about 1g of plant extract and dissolved in pure or distilled water, then filtered. Now the filtrate material was heated with 10 ml of Fehling's solution A and B separately. Red coloured precipitate of cuprous oxide (Cu_2O) formed which shows that the reducing sugars are present.

Test for sterols (Salkowaski reaction)

Few mg of the plant extract was dissolved in 1 ml chloroform, then 1 ml of concentrated sulphuric acid (H_2SO_4) was added. The test tube was vigorously shaken-up for upto 3-4 minutes. Red colour appeared in the chloroform layer, indicating the presence of 'sterols'.

Test for tannins (Ferric chloride reagent test)

The plant extract was taken on an individual basis in pure or distilled water, warm and filtered. Took a little volume of the filtrate and added some drops of 5% w/v solution of ferric chloride, prepared in 90% alcohol. A deep green or blue colour appeared, indicating that the tannins are present.

Test for Saponins

2 g of the plant extract taken and boiled with 10 ml of pure or distilled water, then filtered. Took the filtrate, added about 6 ml of pure or distilled water and vigorously shaken for upto 5 minutes. Foaming which comes on warming indicates the existence of saponins.

Test for terpenoids (Salkowski test)

Took 1 g of extract added 4 ml of chloroform, a further addition of 6 ml of concentrated Sulphuric acid to form a layer. A reddish brown colour of the interface appeared which indicates the presence of terpenoids.

Anthelmintic activity

1. Standard solution

Tinidazole (10 mg/ ml, 25 mg/ ml and 50mg/ ml) was taken as standard solution.

2. Test solution

The different concentrations (10mg/ml, 25mg/ml and 50 mg/ml) of ethyl acetate, chloroform extracts, petroleum ether and methanol extract of the leaves of *Leucaena leucocephala* were prepared. Standard drug tinidazole solution and all the extracts solutions were freshly prepared before starting of the experiments.

3. Experimental design

All the extracts were suspended in 0.5% concentrated solution of tween 80 which is prepared in pure or distilled water. All the solutions and extracts were freshly prepared before the starting of the experiment. 16 groups each were containing 6 earthworms, released into 10 ml of desired formulation as;

Group I were the control worms placed in vehicle 0.5% Tween 80 in distilled water.

Groups II-IV received petroleum ether extracts of *L. leucocephala* at 10 mg/ml, 25 mg/ml and 50 mg/ml concentrations respectively.

Group V-VII treated with chloroform extracts at 10, 25 and 50 mg/ml concentrations respectively.

Group VIII-X treated with the ethyl acetate extract at 10, 25 and 50 mg/ml concentrations respectively.

Group XI-XIII treated with the methanol extracts at 10, 25 and 50 mg/ ml concentrations respectively.

Group 14-16 took as standard which treated with Tinidazole (10, 25 and 50 mg/ ml).

The final volume was set to 10 ml in each of the petridish. Observations were prepared on the basis of the time occupied to paralyse and cause death of each worms individually during the test period. The occurrence of Paralysis was reported when the worms did not survive even in the normal saline. Death was determined when the worms lost motility indicated by fading their body colour.

RESULTS AND DISCUSSIONS

Table No. 1: Preliminary phytochemical analysis of leaf extract of Leucaena Leucocephala.

Phytochemical Tests	Test Used	Petroleum ether	Chloroform	Ethyl Acetate	Methanol
Alkaloids	Mayer's test	++	+	-	+
Anthraquinones		-	++	-	+++
Flavonoids	Ferric chloride test	+	++	+	++
Proteins	Xanthoproteic test	+	+	+	+++
Tannins	Ferric chloride reagent test	++	+++	++	+++
Terpenoids	Salkowaski test	+++	+++	++	+++
Saponins	Foam test	+	-	-	-
Sterols	Salkowaski test	+	-	-	++
Sugars	Fehling's solution test	+++	++	++	+++

Note: slightly Present(+), moderately present(++), Significantly present(+++).

Preliminary phytochemical analysis of all the prepared extracts indicated the presence of anthraquinones, alkaloids, flavonoids, proteins, sugars, sterols, tannins, Saponins and terpenoids. These phytoconstituents are responsible for the anthelmintic activity.

In –vitro Anthelmintic Activity

Effect of different extracts at time of paralysis

Ethyl acetate, chloroform extracts, petroleum ether and methanol extract exhibited better anthelmintic activity when compared with the standard drug Tinidazole at the same concentrations and conditions. Petroleum ether extract occupied minimum time to paralyse the worms followed by Methanol, Ethyl acetate and chloroform respectively when compared with tinidazole drug at 10, 25 and 50 mg/ml dose. The data is clearly shown in Table No.2.

Effect of different extracts at time of death

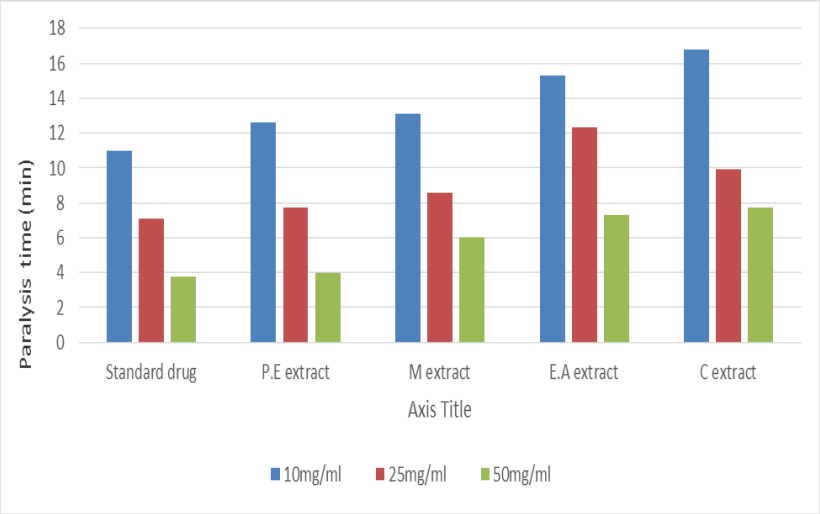
Ethyl acetate, chloroform extracts, petroleum ether and methanol extract exhibited better anthelmintic activity when compared with standard synthetic drug at the same concentration and condition. Petroleum ether extract occupied minimum time to cause death of the worms followed by Methanol, Ethyl acetate and chloroform respectively. If 10 mg/ml dose of tinidazole drug is compared with Chloroform extracts, ethyl acetate, petroleum ether and methanol extract then it can be determined that plant extract contains better effectiveness as compared to synthetic drug for anthelmintic activity. It is clearly shown in Table No.3.

Table No. 2: Anthelmintic activity of different plant extracts of *Leucaena leucocephala* leaves at time of paralysis of earthworms

Treatment	Concentration (mg/ ml)	Paralysis time (min.)
Vehicle (Tween 80)	----	----
Tinidazole	10	11±4.908
	25	7.1±3.807
	50	3.8±1.033
Petroleum ether extract	10	12.6±3.864
	25	**7.7**±1.515
	50	4±1.0
Methanol extract	10	13.1±4.723
	25	8.6±4.219
	50	6±3.226
Ethyl acetate extract	10	15.3±2.507
	25	12.3±1.416
	50	7.3±2.783
Chloroform extract	10	16.8±7.137
	25	9.9±5.826
	50	7.7±7.107

All values represents in mean ± SD; n=6 in each group.

Vehicle worms were alive to 24 hrs under observation.



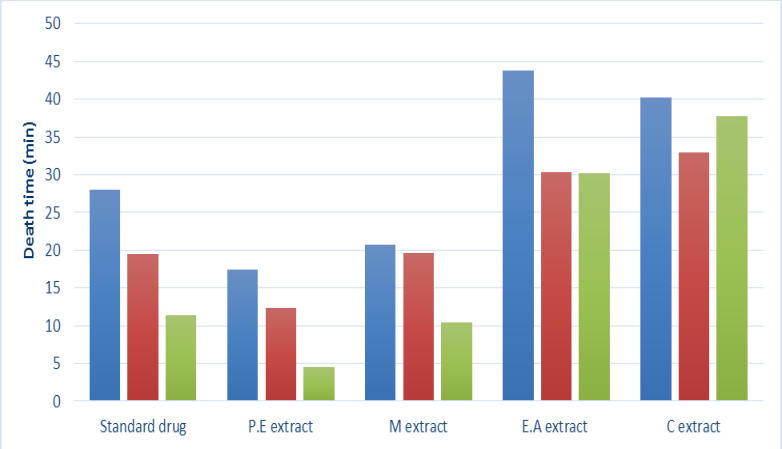
Graph1: Time taken for paralysis after treatment with *Leucaena leucocephala* leaf extracts where C- Chloroform extracts, E.A- ethyl acetate extract, P.E- petroleum ether extract, M- methanol extract and Standard drug- Tinidazole. The number represents paralysis time in minutes.

Table No. 3: Anthelmintic activity of different plant extracts of *Leucaena leucocephala* leaves at the time of death of earthworms.

Treatment	Concentration (mg/ ml)	Paralysis time (min.)
Vehicle (Tween 80)	----	----
Tinidazole	10	28±7.984
	25	19.5±4.692
	50	11.4±4.608
Pet. ether extract	10	17.5±2.309
	25	12.4±2.276
	50	4.5±1.0
Methanol extract	10	20.7±2.634
	25	19.7±3.167
	50	10.5±4.608
Ethyl acetate extract	10	43.8±10.207
	25	30.3±7.416
	50	30.2±8.783
Chloroform extract	10	40.30±9.137
	25	32.9±8.826
	50	37.7±9.307

All values represents in mean ± SD; n=6 in each group.

Vehicle worms were alive to 24 hrs under observation.



Graph2: Time taken for death after treatment with *Leucaena leucocephala* leaf extracts where C- Chloroform extracts, E.A- ethyl acetate extract, P.E- petroleum ether extract, M- methanol extract and Standard drug- Tinidazole. The number represents time in minutes.

Graph 1 and 2 displayed that both petroleum ether extract and methanol extract of leaves exhibited significant anthelmintic activity in dose dependent when compared with reference standard drug Tinidazole. In comparison with methanol extract petroleum ether extract of leaves in concentration of 50 mg/ml was found to be paralysis and death of worm in 4±1.0 and 4.5±1.0 minutes time respectively which is potentially much effective as compare with the standard drug Tinidazole. The compound constituents responsible for anthelmintic activity were not properly investigated however preliminary phytochemical analysis of extracts give positive test for alkaloids, anthraquinones, flavonoids, proteins, sugars, sterols, tannins, terpenoids and Saponins. The role of flavonoids, saponins, alkaloids and steroids are responsible for anthelmintic activity. The comparison between treated groups with standards was carried out using one way ANOVA test. All the results were found to be significant with P value less than 0.0001 ($P < 0.0001$).

DISCUSSION

The data revealed that the various extracts showed paralysis and time of death at a concentration of 50 mg/ ml, 25 mg/ml, 10 mg/ml in concentration dependent manner. The test concentration of all the extracts showed marked degree of anthelmintic activity with maximum activity of petroleum ether extract. The anthelmintic effect of extract is compared to the effect produced by the standard drug tinidazole. Marvellous researches have been done during the previous decade and large numbers of synthetic precursors have been derived to cover the damage caused by parasites. But unfortunately no effective medicine has been developed till now. Some severe side effects of drug and development of resistance increases

the severity of infection to the next level. These factors covered the way for herbal remedies as alternative anthelmintic. The result of this study has shown promising anthelmintic activity suggesting the possible use of *Leucaena leucocephala* extract in control of intestinal nematode.

CONCLUSION

This study has demonstrated that the various leaf extract of *Leucaena leucocephala* possess significant in vitro anti-worm activity at the tested concentrations. The petroleum ether extract shows maximum activity at all the tested concentration. Thus, the wormicidal activities of the plant extract against earthworms suggest that it can be effective against parasitic helminths of humans and animals. However, further studies are needed to isolate, characterize and evaluate the actual bioactive components and their mechanism of action. Also, studies on the toxicity, evaluation of the effect in-vivo and the establishment of adequate doses for human and animals are recommended.

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