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PRELIMINARY PHYTOCHEMICAL SCREENING, *IN-VITRO* EVALUATION OF *TRIFOLIUM REPENS* L. FOR ANTHELMINTIC AND ANTIBACTERIAL ACTIVITIES

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ABSTRACT

The present work was conducted to investigate the preliminary phytochemical studies, antihelminthic and anti-bacterial activities on the leaf extracts of Trifolium repens L. family Fabaceae. The anthelmintic activity was evaluated on adult Indian earthworm Pheritima posthuma due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings. The antibacterial activities of the extracts of Trifolium repens L. were performed by agar cup plate method. Preliminary phytochemical screening of petroleum ether, ethyl acetate, methanol and aqueous extracts of leaves of T. repens L. revealed the presence of carbohydrates, saponins, phytosterols, triterpenoids, tannins, phenolic compounds, alkaloids and amino acids. All the tested extracts showed mild to moderate anthelmintic activity and antimicrobial activity, albendazole and amikacin sulfate were employed as reference standard for anthelmintic and antibacterial activities respectively. Among the tested extracts the aqueous and methanolic extracts were found to possess potent anthelmintic activity while ethyl acetate extract showed moderate activity. Whereas petroleum ether extract showed most promising antibacterial activity, while ethyl acetate showed moderate activity. The present study shows the potent anthelmintic as well as antibacterial activity of T. repens L. that was good in their action against the worms.

KEY WORDS

Anthelmintic activity, antimicrobial activity, phytochemical studies and Trifolium repens L.

INTRODUCTION

Medicinal plants have served through ages, as a constant source of medicaments for the treatment of a variety of diseases. The history of herbal medicine is almost as old as human civilization. The plants are known to provide a rich source of botanical anthelmintic, antibacterial and insecticide. [1] Helminth infections are among the most common infections in man, affecting a large proportion of the world's population. In developing countries, they pose a large threat to public health and contribute to the prevalence of malnutrition, anaemia, eosinophilia and pneumonia. Although the majority of infections due to worms are generally limited to tropical regions, they can occur to travelers who have visited those areas and some of them can develop in temperate climates. A parasitic disease causes severe morbidity, including lymphatic filariasis (a cause of elephantiasis), onchocerciasis (river blindness) and schistosomiasis. These infections can affect most populations in endemic areas with major economic and social consequences. [2]

Trifolium repens L. the white clover is also known as Dutch clover, Ladino clover or ladino and belongs to the family Fabaceae is cultivated throughout World. The plant is considered as anti-rheumatic, depurative, anti-scrophulatic and tonic, the tincture of leaves is used as ointment for gout, the flower infusion is used as eye wash. In Turkish folk medicine, it is used as an

expectorant, antiseptic, analgesic properties and treatment for rheumatic aches. [3] In India, *T. repens* is considered a folk medicine against intestinal helminthic worms and an experimental *in-vivo* study validated that the aerial shoots of *T.repens* bear significant anticestodal properties.[4]

The present study reports the preliminary phytochemical studies, anthelmintic activity and antibacterial activity on the leaf extract of *T. repens* L.

MATERIALS AND METHODS

Plant material

The plant material was collected from medicinal garden of Venkateshwara Institute of Pharmaceutical sciences, located at cherlapally, Nalgonda District, Telangana state, during the month of November-December 2017. After authentication the collected leaves were cleaned thoroughly and dried under the shade. Once the drying process is completed, the dried leaves were ground to powder using blender for further use.

Preparation of extracts

The powdered leaf material of *Trifolium repens* L. was subjected to successive solvent extraction with methanol. 40gm of powdered leaf material was subjected to soxhlet extraction for about 10 hours with 350 ml of the methanol solvent. The extracts obtained were later kept for distillation to remove the excessive solvent. These extracts were mixed and dried. The aqueous extract was fractioned by using different solvents like ethyl acetate and petroleum ether and these extracts were stored in a cool and dry place for further analysis.

Preliminary phytochemical analysis [4]

During preliminary phytochemical screening tests were mainly concluded to alkaloids, amino acids, carbohydrates, flavanoids, saponins, steroids, triterpenoids, proteins, tannins and phenolic compounds. The constituents are reported in table no **(1)**.

Thin Layer Chromatography

Slurry of silica gel G was prepared in distilled water and poured on glass plate to form a thin film. The prepared plates were allowed for setting (air-drying). After setting, the plates were kept in an oven at 100 to 120^{0C} (30 min) for activation. The extracts of samples were applied to the activated plates (1cm above from the bottom). It was then kept in previously saturated developing chamber containing mobile phase and allowed to run 3/4th of height of the plate. Mobile phase was previously saturated in a chamber, and ascending development technique is commonly used. TLC plates were placed in iodine chamber for spot location. Rf Values were calculated by noting distance travelled by solute and solvent.

Anthelmintic activity [5]

The anthelmintic activity was evaluated on adult Indian earthworm *Pheretima posthuma* (Annelida). It resembles anatomically and physiologically with the intestinal round worm parasite of human being. The solvent extract was dissolved in a minimum amount of Di-methylsulphoxide (DMSO) and the volume was adjusted with normal saline water. The *in-vitro* tests were started within one hour after collection of the organism. Each group consists of 6 earthworms. Indian adult earthworms, collected from moist soil and washed with normal saline to remove all faecal matter, were used for anthelmintic activity. Different concentrations of the dried extracts (25, 50 mg/ml) were prepared. 5ml of each concentration of aqueous extract was delivered into a petridish. Then six worms (same type) were placed in it.

Similarly, for each concentration of methanolic extract, six worms were used. Time for paralysis was noted when the worm did not revive even in normal saline. Time for death of warms were also recorded when the worms lost their motility followed by fading away of their body colour. Albendazole (10mg/ml in distilled water) was used as positive control.

Anti-bacterial activity [6]

The antibacterial activities of the crude extracts were performed by agar cup plate method. The extracts were dissolved in DMSO at a concentration of 50 and 100 µg/ml respectively. Amikacin sulfate (50 and 100 µg/ml) in DMSO was used as reference standard for the antibacterial activity. The selected microorganisms include B. subtilis and E. coli.

RESULTS AND DISCUSSION

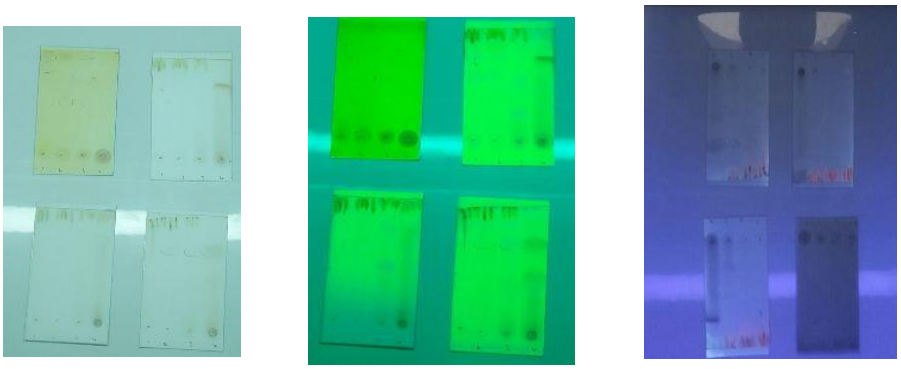
Phytochemical Screening

Preliminary phytochemical screening of petroleum ether, Ethyl acetate (EtOAc), Methanol (MeOH) and aqueous extracts of leaves of *Trifolium repens* L. revealed the presence of carbohydrates, saponins, phytosterols, triterpenoids, tannin, phenolic compounds, alkaloids and amino acids. Thus, the

preliminary phytochemical tests are helpful in finding chemical constituents in the plant material that may lead to their quantitative estimation and also in locating the source of pharmacologically active chemical compounds. Findings were tabulated in table no (1).

Thin Layer Chromatography

The aqueous, methanolic and ethyl acetate extracts of *Trifolium repens* L. were subjected for TLC and their Rf values are given in Table no (2). Aqueous extract showed four spots at 0.92, 0.88, 0.73 and 0.72. Methanolic extract also showed four spots at 0.87, 0.85, 0.66 and 0.5. Ethyl acetate extract showed five spots at 0.88, 0.85, 0.72, 0.64 and 0.5. Petroleum extract showed five spots at 0.92, 0.85, 0.85, 0.80 and 0.61. The second spot (R_f 0.85) of methanolic extract was matching with the second spot of ethylacetate extract and third & fourth spot of petroleum ether extract. The first spot (R_f 0.92) of aqueous extract was matching with the first spot of petroleum ether extract and the fourth spot (R_f 0.5) of methanolic extract and also matching with fifth spot of ethylacetate extract. Hence, the R_f values states that some of the chemical components are similar in all the extracts.



a **b** **c**

Fig.1 TLC Profile of Extracts

a. Visible light, b. short Wavelength (274 nm) c. Long Wavelength (375 nm)

Antihelmintic activity

All the extracts were tested for antihelmenthic activity; albendazole was employed as reference standard. It has been observed that all the tested extracts showed mild to moderate antihelmenthic activity.

Among the tested extracts the aqueous and methanolic extracts were found to possess potent anthelmintic activity while ethyl acetate extract showed moderate activity. Table no. (3) shows the results of the test performed.

Antibacterial studies

All the extracts were tested for *in-vitro* antibacterial activity by the agar cup plate method and the zone of inhibition values are presented in table no. (4).

Amikacin sulfate was employed as reference standard to compare the results. It has been observed that all the tested extracts showed mild to moderate activity against the bacteria. Whereas petroleum ether extract showed most promising antibacterial activity, while ethyl acetate showed moderate activity.

Table No. 1: Qualitative preliminary phytochemical screening

Test	Aqueous extract	Methanolic extract	Ethyl acetate extract	Petroleum ether extract
1.Alkaloids test: Dragendorff's test:	++	++	–	–
2.Aminoacid test: Ninhydrin test:	++	+	–	–
3.Carbohydrates test: Barfoed test:	–	–	–	+
4. Test for flavanoids: Shinoda test:	+++	+++	+	–

Test	Aqueous extract	Methanolic extract	Ethyl acetate extract	Petroleum ether extract
5. Test for Saponin: Foam test:	++	+	+	+
6.Glycosides test: Keller killani test:	–	–	–	–
7.Steroids test: Liebermann buchard test:	–	+++	++	+
8.Terpenoids test: Liebermann burchard test:	++	–	–	–
9.Tannins & Phenolic compounds test: Ferric chloride test:	+++	+++	++	–

"+ Slight changes, ++ moderate, +++ stronger reactions,"

Table No. 2: TLC profile of extracts of *Trifolium repens L.* & Rf values

S No.	Extracts	TLC Spots	Rf Values
1	**Aqueous extract**	Spot 1	0.92
		Spot 2	0.88
		Spot 3	0.73
		Spot 4	0.72
2	**Methanol extract**	Spot 1	0.87
		Spot 2	0.85
		Spot 3	0.66
		Spot 4	0.5
3	**Ethyl acetate extract**	Spot 1	0.88
		Spot 2	0.85
		Spot 3	0.72
		Spot 4	0.64
		Spot 5	0.5
4	**Petroleum ether extract**	Spot 1	0.92
		Spot 2	0.85
		Spot 3	0.85
		Spot 4	0.80
		Spot 5	061

Table No. 3: Antihelminthic activity of extracts of *Trifolium repens.L*

S.No	Drug Treatment	Dose (mg/ml)	Time of paralysis (min)	Time of death (min)
1	Control	-	-	-
2	Albendazole	10	72	123
3	Aqueous extract	20	4	18
		50	2	8.4
4	Methanolic extract	20	8	27.34
		50	5	14.56
5	Ethyl acetate Extract	20	23.30	43.24
		50	12.2	31

Table No.4: Antibacterial activity results for the extracts

Extracts	Zone of inhibition (in mm) Quantity in µg/ml			
	B.subtilis		*E.coli*	
	50	**100**	**50**	**100**
Aqueous Extract	11	12	10	13
Petroleum Ether	10	17	16	19
Ethyl Acetate	10	11	15	20
Amikacin sulfate	28	33	25	28

CONCLUSION

In the present study, all the extracts of leaves of *Trifolium repens L.* were evaluated for anthelmintic and antibacterial activities *in vitro*. From the above results it was clear that almost all the extracts of leaves of *T. repens L.* have certain potent anthelmintic and antibacterial activity as compared to standard drugs. Further studies are required to identify the actual chemical constituents that are present in the crude drug extracts of this plant which are responsible for anthelmintic and antibacterial activity.

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