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ANTIBACTERIAL ACTIVITY AND HPLC ANALYSIS OF *THEVETIA PERUVIANA* LEAF EXTRACTS

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ABSTRACT: *Thevetia Peruviana* (or *cascabela thevetia*) belongs to the family Apocynaceae and commonly called as Yellow Oleander. The leaves of this significant medicinal plant were subjected to antibacterial investigation and HPLC analysis. Hexane, acetone and methanol were used as organic solvents. Four different concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml) of each extract *i.e.* hexane, acetone and methanol were prepared for antibacterial activity by dissolving them in 100% DMSO. The leaf extracts were subjected to test the antibacterial activity by agar well diffusion method. Methanol extract of leaves showed significant antibacterial activity against *Pseudomonas aeruginosa* and poor activity against *Micrococcus luteus* and *Shigella flexneri.* Acetone extract showed moderate activity against the tested micro-organisms *viz. Pseudomonas aeruginosa, Micrococcus luteus* and *Shigella flexneri*. Hexane extract showed moderate activity against *Micrococcus luteus* and poor activity against *Pseudomonas aeruginosa and Shigella flexneri* at different concentrations. HPLC analysis of methanol leaves extract was performed which showed that plant leaf contains different types of phytosterols. *Thevetia peruviana* leaves offers promising possibility of developing a new drug of pharmaceutical interest.

INTRODUCTION: The use of medicinal plants as a source of relief from illness can be traced back over five millennia to written documents of the early civilization in China, India and the Near East. The potential of higher plants as source for new drugs is still largely unexplored. Among the estimated 250,000 - 500,000 plant species, only a small percentage has been investigated phyto-chemically and the fraction submitted to biological or pharmacological screening is even smaller. Historically pharmacological screening of compounds of natural or synthetic origin has been source of innumerable therapeutic agents.

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Random screening as tool in discovering new biologically active molecules has been most productive in the area of antibiotics. Plant based antimicrobials represent a vast untrapped source for medicines. They are effective in the treatment of infectious diseases while simultaneously mitigating some of the side effects of synthetic antimicrobials. They may act as lead compounds for the pharmaceutical industry or as the base for the development of new antimicrobials.

Thevetia peruviana (yellow oleander) belongs to the family Apocynaceae and is an evergreen Indian tropical shrub that bears orange – yellow flowers. *Thevetia peruviana* is cultivated as an ornamental plant. Mostly all parts of plant *i.e.* leaf, seed, flower, fruit and root are of significant medicinal value. The leaves of this plant contain flavanone and flavanol glycosides which showed inhibitory activities against HIV-1 reverse transcriptase and HIV-1 integrase [1].

Various other bioactivities of *Thevetia peruviana* such as antispermatogenic [2], piscicidal [3], anti-termite [4], antifungal [5 – 6], antidiarrheal, antimicrobial and cytotoxic [7], anti-inflammatory [8] have been reported. The whole plant is toxic and poisonous [9 - 10]. The present study was undertaken to evaluate the antibacterial activity of *Thevetia peruviana* leaves extracts by agar well diffusion method [11] and chemical profiling of the same by HPLC (High Performance Liquid Chromatography) for the presence of phytosterols.

MATERIALS AND METHODS:

Collection of Plant Materials: Fresh leaves of *Thevetia peruviana* were collected randomly from Jaipur, Rajasthan and identified and authenticated in the herbarium unit of Department of Botany, Rajasthan University and a voucher specimen (RUBL211530) has been deposited.

Plant Extract Preparation: The leaves were properly washed with tap water and then rinsed with distilled water. The rinsed leaves were shed dried for 1 week. The crispy leaves were then reduced to powder using a blender and then stored in air tight bottles. The powdered plant material (100gm × 3) was extracted with hexane, acetone and methanol by continuous hot extraction using soxhlet apparatus at a temperature not exceeding the boiling point of the solvents. The extracts were then filtered and evaporated on a water bath at low temp. (40 °C - 50 °C) to a syrupy consistency and then to dryness. The hexane, acetone and methanol dry extract weight 3 gm, 5.2 gm and 8.6 gm respectively. The extracts so obtained were stored at 4 °C until used.

Antibacterial Activity: The hexane, acetone and methanol crude extracts of *Thevetia peruviana* leaves were used for the determination of antibacterial activity against three pathogenic bacterial strains. The extracts were prepared for antibacterial activity by dissolving them in 100% DMSO and four concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml) of each extract *i.e.* hexane, acetone and methanol were prepared.

Microorganisms Used: Bacterial cultures were procured from MTCC, Institute of Microbial Technology, Chandigarh *viz. Micrococcus luteus* (MTCC code 2848), *Pseudomonas aeruginosa* (MTCC code 1688) and *Shigella flexneri (*MTCC code 1457).

Growth and Maintenance of Test Micro-organisms: The bacterial cultures were grown in nutrient agar medium and incubated at 37 °C (*P. aeruginosa and S. flexneri*) for 24 hrs and 30 °C (*M. luteus*) for 48 hrs. Each bacterial culture was further maintained on the same medium after 24 hrs and 48 hrs of transferring the respective bacteria. A fresh suspension of test organisms in saline solution was prepared from a freshly grown agar slant before every antibacterial assay.

Determination of Antibacterial Activity: *In vitro* antibacterial activity was studied against the above mentioned bacterial strains by the agar well diffusion method. The nutrient agar was melted and cooled to 48 °C – 50 °C and a standarized inoculum (1.5×10^8 CFU/ml, 0.5 Mc Farland) was then added aseptically to the molten agar and poured into sterile petridishes to give a solid plate. Wells were prepared in the seeded agar plates. The test compounds were administered to fullness in each well 6 mm in diameter and allowed to stand on the bench for 1hr. for proper diffusion. The plates were then incubated at 37 °C for 24 hrs for *P. aeruginosa* and *S. flexneri* and 30 °C for 48 hrs for *M. luteus*. The antibacterial spectrum of the test compounds was determined for the bacterial species in terms of zone sizes around each well. The diameter of the inhibition zone was measured in mm. The experiment was performed in triplicate to minimize the error and the mean values were calculated. Ampicillin and Amikacin used as positive control.

Preparation of Sample for HPLC: 10 mg of methanolic leaves extract of *T. peruviana* was dissolved in 10 ml of HPLC grade methanol to get final conc. of 1 mg/ml. Prior to injection in the sample loop, the extract was degassed in an ultrasonic bath and filtered using 0.45 µm syringe filter (millipore) for sterilization.

Procedure of HPLC: Binary system equipped with PDA detector connected to system processor was used for analysis. A maximum pressure of 2500 psi and minimum of 1500 psi was maintained. The solvent was run at 202 nm wavelength using reverse phase C-18 column. During the run, a flow

rate of 1 ml/min. was maintained and the injection volume was 10 µl.

RESULTS:

Antibacterial Activity: The antibacterial activity of hexane, acetone and methanol extract of *T. peruviana* leaves were tested against 3 bacterial strains *i.e. Micrococcus luteus, Pseudomonas aeruginosa* and *Shigella flexneri*. Individually, against *Micrococcus luteus* hexane extract 100 mg/ml showed inhibition zone 12 mm. Acetone extract 100 mg/ml and 25 mg/ml showed inhibition zone 12 mm and 10 mm respectively. Methanol extract 200 mg/ml and 100 mg/ml both showed inhibition zone 6 mm.

In case of *Pseudomonas aeruginosa* methanol extract 200 mg/ml, 100 mg/ml and 50 mg/ml showed inhibition zone 14 mm, 26 mm and 16 mm respectively. Acetone extracts 200 mg/ml, 100 mg/ml and 25 mg/ml showed inhibition zone 10 mm, 8 mm and 8 mm respectively. Hexane extract showed activity only at 25 mg/ml, measured 8 mm. Against *Shigella flexneri* acetone extract 200 mg/ml, 100 mg/ml and 25 mg/ml showed inhibition zone 12 mm, 6 mm and 10 mm respectively. Methanol extract 100 mg/ml showed inhibition zone 8 mm. Hexane extract showed inhibition zone of 4 mm at 50 mg (**Table 1)**.

TABLE 1: ANTIBACTERIAL ACTIVITY OF *THEVETIA PERUVIANA* LEAF EXTRACTS

Test Organisms	Zone of Inhibition												
	HE (mg/ml)				AE (mg/ml)				ME (mg/ml)				AM/AK
	200	100	50	25	200	100	50	25	200	100	50	25	
Micrococcus luteus	NA	12± 0.39	NA	NA	NA	12± 0.23	NA	10± 0.53	06± 0.35	06± 0.71	NA	NA	21± 0.23 (AM)
Pseudomonas aeruginosa	NA	NA	NA	8± 0.11	10± 0.41	8± 0.58	NA	8± 0.62	14± 0.27	26± 0.31	16± 0.73	NA	22± 0.34 (AK)
Shigella flexneri	NA	NA	4± 0.31	NA	12± 0.49	6± 0.33	NA	10± 0.16	NA	8± 0.12	NA	NA	22±0.13 (AK)

HE – Hexane extract, AE – Acetone extract, ME – Methanol extract; AM – Ampicillin; AK – Amikacin; Inhibition zone in mm, Mean ± SE; NA - No Activity.

HPLC Analysis: HPLC 'fingerprint' (**Fig. 1** and **Table 2)** of the methanolic extract of *Thevetia peruviana* leaves showed seven peaks with retention times (min.) 1.6, 2.3, 2.4, 3.1, 6.3, 7.5 and 10.3 at the wavelength of 202 nm. HPLC analysis of the sample revealed the presence of multiple phytosterols.

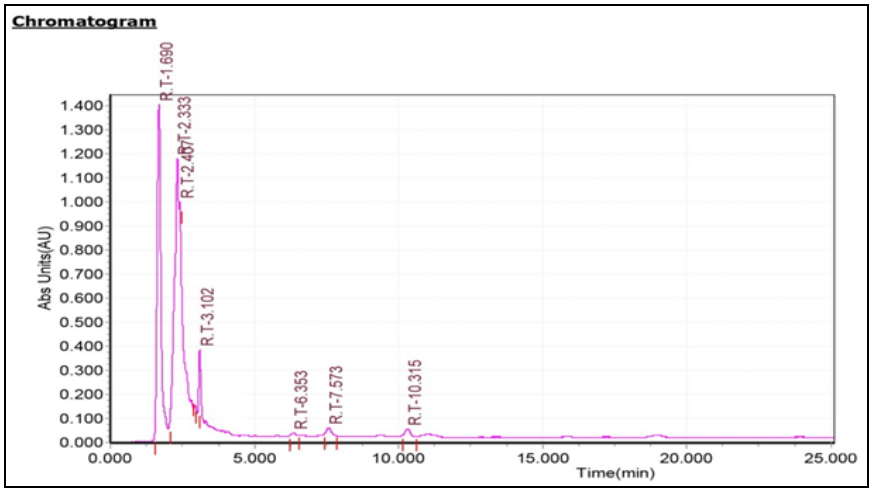

FIG. 1: HPLC PEAKS

TABLE 2: HPLC PEAK INFO

Peak	R.T. (min.)	Height%	Area%
1	1.690	63.273	64.346
2	2.333	15.310	5.292
3	2.407	4.689	18.146

4	3.102	13.298	7.965
5	6.353	0.771	0.508
6	7.573	1.230	1.632
7	10.315	1.429	2.111

DISCUSSION: Plants synthesize variety of phytochemicals as part of their metabolic activities. It has been widely observed and accepted that the medicinal value of plants lies in these phytochemicals. Chemical profile of a single plant may vary over time as to changing conditions. Plant scientists and natural products chemists are combing the flora for the phytochemicals, which could be developed for the treatment of various diseases.

In the present investigation the antibacterial activity of different solvent extracts (hexane, acetone and methanol) of *Thevetia peruviana* leaves was done by agar well diffusion method against three pathogenic bacterial strains. It was found that different solvent extracts exhibited appreciable to no activity at all against the tested organisms. The hexane extract showed moderate activity against *Micrococcus luteus* at 100 mg/ml, inhibition zone measured 12 mm. Against *Pseudomonas aeruginosa* 25 mg/ml showed a inhibition zone of 8 mm. The results were poor for *Shigella flexneri*.

The methanol extract showed appreciable activity against *Pseudomonas aeruginosa* at 100 mg/ml, inhibition zone measured 26 mm. Also at 200 mg/ml and 50 mg/ml the activities were significant. Against *Shigella flexneri* an inhibition zone of 8mm was measured at 100 mg/ml. The activity was poor against *Micrococcus luteus*. In case of acetone extract for *Micrococcus luteus* 100 mg/ml showed an inhibition of 12 mm whereas for *Shigella flexneri* 200 mg/ml showed the same inhibition zone of 12 mm. At 200 mg/ml an inhibition zone of 10 mm was measured for *Pseudomonas aeruginosa.*

In the present study HPLC analysis of methanolic leaves extract was also undertaken. The study revealed that *Thevetia peruviana* leaves do contain some phytosterols but is not rich source of phytosterols.

CONCLUSION: The importance of medicinal plant research is that it has helped to establish the efficacy and safety of traditional medicine and plant products.

The phytochemicals present have shown immense importance in relieving the society from various diseases. This has also helped in synthesizing isolated analog molecules by the pharmaceutical industry, in the run towards finding new, cheap and novel drugs.

This study provides scientific information about *Thevetia peruviana*, based on its antibacterial potential and chemical profiling. The antibacterial activity of *Thevetia peruviana* leaves may be attributed to various phytochemical constituents present in the crude extract. The purified components may have even more potency with respect to inhibition of microbes. Thus, there is still a lot of scope for further research which can reveal the exact potential of the plant to inhibit pathogenic microbes.

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