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Phytochemical Screening and *in Vitro* Antimicrobial Investigation of the Methanolic Extract of *Xanthium strumarium* Leaf

P. Srinivas[1*] V. Rajashekar[2*], Upender Rao. E, Venkateshwarulu. L and Anil kumar CH
[*2]Department of Pharmaceutical Chemistry, Vikas college of Pharmacy Vikas nagar, Shameerpet Mdl. Jangaon; Dist: Warangal A.P (India)
[*1]Department of pharmaceutical Microbiology. Vikas college of Pharmacy, Vikas nagar, Shameerpet Mdl. Jangaon; Dist: Warangal A.P (India)

Abstract

Extract in organic solvent methanol of *X. strumarium* evaluated for their Phytochemical screening and antibacterial activities against eight pathogenic bacteria such as, *Klebsiella pneumoniae* NCIM 2719, *Proteus vulgaris* NCTC 8313, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas putida* ATCC 12842, *Salmonella typhimurium* ATCC 23564, *Bacillus cereus* ATCC 12842, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* ATCC 12228. It was carried out by taking the Methanolic extracts of leaf at a concentration of 50 mg/ml and100 mg/ml their activities were recorded by estimating zones of inhibition as produced by disc-diffusion method on Mueller-Hinton agar media. Based on the activity and preliminary phytochemical analysis TLC and bioautography with different solvent systems resoluted for compounds at different R_F valued simultaneously TLC- bio-autography was performed to determine active compounds R_F based on TLC.

*Corresponding author, Mailing address:
Pogu Srinivas
Associate Professor
Department of Pharmaceutical Microbiology
Vikas College of Pharmacy
Vikas nagar, Shameerpet
Mdl. Jangaon; Dist: Warangal A.P (India).
Email: cnupogu@yahoo.co.in

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Antibacterial activity, *X. Strumarium*, TLC, Bioautography, Phytochemicals

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Introduction

Medicinal plants are important therapeutic aid for various ailments. There is wide spread interest in drugs derived from plants. This interest primarily stems from the belief that green medicine is safe and

dependable. The production of medicines and the pharmacological treatment of diseases began with the use of herbs [1]. Some of the effects elaborated by extract of plants used in traditional medicine include antiviral, antitumor, antimicrobial, insecticide, etc. As a part of antibacterial production plants have many ways of generating antibacterial compounds to protect them against pathogens[2]. Nature has been source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. Many medicinal plants are used in modern medicine where they occupy a very significant place as raw materials for important drugs and plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases.

X. strumarium is an annual herb with a short, stout, hairy stem. Leaves broadly triangular-ovate or suborbicular; flower heads in terminal and axillary racemes; white or green; numerous; male upper most; female ovoid, covered with hooked bristles; Fruit obovoid, enclosed in the hardened involucre, with 2 hooked beaks and hooked bristles. Flowering time in India is August-September. It can be propagated through seeds. This weed is easily dispersed through animals as the fruits have hooked bristles and 2 strong hooked beaks [3].

The whole plant, specially root and fruit, is used as medicine. According to Ayurveda, *X. strumarium* is cooling, laxative, fattening, anthelmintic, alexiteric, tonic, digestive, antipyretic, and improves appetite, voice, complexion, and memory. It cures leucoderma, biliousness, and poisonous bites of insects, epilepsy, salivation and fever. The plant of *Xanthium* yields xanthinin which acts as a plant growth regulator. Antibacterial activity of xanthinin has also been reported. Seed yields semi-drying edible oil (30-35%) which resembles sunflower oil and used in bladder infection, herpes, and erysipelas. Cake can be used as manure whereas shell can be used as activated carbon[4,5]. The plant has been reported as fatal to cattle and pigs.

Materials and Methods

The investigations embodied in this thesis were carried out in the Department of Microbiology, Vikas College of pharmacy, and Jangaon. Laboratory experiments are carried out under uniform laboratory conditions. Field experiments were conducted in the experimental fields attached to the Department. Throughout the investigations, whenever requires and unless otherwise mentioned Merck or sigma analytical grade chemicals are used. The glassware was Merck or Borosil mark. It was thoroughly washed in chromic acid and rinsed with distilled water before the use for experiment. Standard methodology recommended by various investigators was employed in these investigations. The data obtained in these investigations was subjected to statistical analysis. The interferences are drawn on my own observations and also taking the help of valuable information available on the subject.

Plant Material

Based on ethnomedical information and literature surveys the following plants were selected for study. *X. strumarium* leaves were collected from rural areas of Jangaon, Warangal Dist, AP, India. The taxonomic identities of these plants were confirmed by department of Botany, A.B.V Govt. Deg. College and Jangaon, India. A brief description of the and systemic position is provided with: [6].

Fresh leaves of plant free from diseases were collected and bought to laboratory in sterile polyethylene bags and washed thoroughly 2-3 times with running tap water and then once with sterile water, shade dried for two weeks, subsequently ground into fine powder using mechanical grinder and motor driven grinding mill . The powder was used for extraction of crude extracts.

Preliminary Phytochemical analysis

Preliminary phytochemical screening of the extracts for saponins, glycosides, alkaloids, tannins and flavonoids using standard phytochemical screening methods [7].

Antimicrobial Assay

The microbial strains used in the present study were obtained from National Chemical Laboratory (NCL). The bacterial strains comprised: *Klebsiella pneumoniae* NCIM 2719, *Proteus vulgaris* NCTC 8313, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas putida* ATCC 12842, *Salmonella typhimurium* ATCC 23564, *Bacillus cereus* ATCC 12842, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* ATCC 12228.

Preparation of inoculums

The bacteria were maintained in Mueller-Hinton Agar (MH). Inocula were prepared by adding an overnight culture of the organisms in Nutrient broth to obtain an OD_{600} 0.1. The cells were allowed to grow until they obtain the McFarland standard 0.5 (approximately 10^8 CFU/ml). The suspensions were then diluted to 1:100 in Nutrient broth to obtain 10^6 CFU/ml.

The medium was prepared by dissolving all the ingredients in distilled water and subjected to sterilization in an autoclave at/121 °C for 15 minutes. The Petri plates were washed thoroughly and sterilized in hot air oven at 160 °C for 1 ½ hours. 30 ml of sterile molten agar medium was seeded by organisms (about 2 ml according to Mc Farland's standard), in semi hot conditions (40 °C) was poured aseptically in sterile Petri plate and allowed to solidify at room temperature. Bores were made on the medium using sterile borer and 0.1 ml of the extracts at 50 and 100 mg/ml concentration in DMSO were added to respective bores and 0.1ml of the standard Amikacin at a concentration of 5µg/ml was used as standard. The Petri plates seeded with organisms, containing extracts and the standard were kept in a refrigerator at 4 °C for 1 hour to facilitate the diffusion of the extracts and the standard in to the media. After diffusion, the Petri plates were incubated at 37 ± 10 °C for 24 hours in an incubator and later the zone of inhibition was observed and measured using a scale [8].

Mueller Hinton Agar (MHA)

Component	g/l
Beef, dehydrated infusion	2.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH at 25 °C	*7.4 ± 0.2*

Nutrient Broth (NB)

Component	g/l
Beef extract	3.0
Sodium chloride	5.0
Yeast extract	1.5
Peptone	5.0
Final pH at 25°C	*7.2 ± 0.2*

Statistical analysis

Diameter of zone of inhibition (excluding well diameter) resulted from replicates were expressed as mean ± standard deviation (SD). The data were analysed by one-way analysis of variance (ANOVA), P value < 0.05 was considered as significant and mean values were compared by using Least Significant Difference (LSD) test using computer software, SAS system for windows (version 8).

Thin Layer Chromatography (TLC)

Each of the aforesaid eight active fractions was, to begin with, checked by Thin Layer Chromatography (TLC) on analytical plates over silica gel (TLC-grade; Merck India) with appropriate mobile phase by trial and error method for determination of mobile phase.

Preparation of the TLC chamber

The developing solvent is placed into a TLC chamber. The solvent completely covered the bottom of the

chamber to the depth of approximately 0.5 cm. The chamber is closed and shaken. It is kept covered so that evaporation doesn't change the composition of developing solvent mixture. After 15 minutes the chamber is saturated with the solvent vapor.

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Experimental procedure

The loaded TLC plate is carefully placed in the TLC chamber with the sample line toward the bottom. The plate whose top is leaned against the jar wall adjusted to sit on the bottom of the chamber and in contact with the developing solvent (solvent surface is below the extract line). The TLC chamber is covered and the plate is allowed to remain undisturbed. When the solvent front has reached three quarters of the length of the plate, the plate is removed from the developing chamber and the position of the solvent front is immediately marked. The plate is allowed to air dry until solvent has removed and then placed in iodine chamber for visualizing spots.

$$R_f = \frac{\textit{Distance traveled by the compound}}{\textit{Distance traveled by the solvent front}}$$

Bio-autography

Plant extracts (µ*l*) were applied at 2.5 cms from the base of pre coated TLC Plate (60 F254) After drying, the TLC plates were developed in suitable mobile phase and run in duplicate, one set was used as the reference chromatogram and other set was used for bio-autography.

The reference TLC plates were then developed and visualized under visible and UV light at 254 and 366 nm [9]. The chromatogram so developed as described above, was placed in TLC plates (10×20 cm) with bacterial culture at 10^6 CFU/ml in molten Mueller Hinton agar was distributed over TLC Plates. After the solidification of the medium, the TLC plate was incubated overnight at 37°C subsequently. Bioautogram developed was sprayed with 1% aqueous solution of 2, 3, 5, triphenyl tetrazolium chloride (TTC) and incubated at 37°C for four hours. Inhibition zone indicated the presence of active compounds [10].

Results and Discussions

Recently, much attention has been directed towards extracts and biologically active compounds isolated from popular plant species. The use of medicinal plant plays a vital role covering the health needs in developing countries, and these plants may offer a new source of antibacterial, antifungal and antiviral agents with significant activity against infective bacteria. (11, 12).

Results from our preliminary phytochemical analysis in methanolic extract of leaves revealed the presence of high concentration of alkaloids, phenolic acid and diterpenes, while, moderate concentrations of glycosides, Saponins, Phytosterols, fixed oils, but negative results were obtained for the carbohydrates, resins, phenols, tannins, Flavonoids and proteins. table-1. The presence of phytochemicals varies based on the solvents used, climatic conditions of plant grown and preliminary phytochemical screening methods.

An extensive survey and interaction with local ethanopharmacologists, herbal drug sellers and rural negative healers revealed that native plant *X. strumarium* are widely used for treatment of various ailments of human beings and live stock. Naturally, it insisted us to verify the traditional wisdom of local community in the use of this plant leaves as herbal

drug. Though, wide range of applications we focused on the antibacterial activity of leaves.

The biologically active compounds correlated to known substances that possess antibacterial properties. (13). Methanolic extract of *X. strumarium* leaves showed different activities against eight pathogenic bacterial strains. The data reported (table-2) presents the antibacterial activity of methanolic extract of leaves was active against 100% bacteriae used.

The antibacterial activity of methanolic extract of leaves exhibited significant against two concentrations used (50 and 100 mg/ml).

The *P.putida and K.pneumoniae* were shown highest activity of (15mm) at both concentrations used in our studies, followed by *P.aeruginosa*, *P. typhi* exhibited 13mm at 100 mg/ml and 11mm at 50mg/ml, *Proteus vulgaris*, *B. cereus* and *S.epidermidis* shown promising activity of an average of 12mm zone of inhibition at 100mg/ml concentration where as 10mm at 50mg/ml. It is clearly observed from data obtained from the table-2 that, from our study there is no much variance of activity shown by two concentrations used. This might be due to the bacteriostatic effect of active phytochemical constituents present in leaves. The activities of methanolic extract of leaves were correlated against standard drug Amikacin at the concentration of 5mg/ml.

Amongst the gram-positive and gram-negative bacteria, gram-positive bacterial strains were more susceptible to extracts as compared to the gram-negative bacteria [14, 15]. But, in our studies the all organisms were sensitive to the methanolic extract of *Xanthium strumarium*.

Besides, additional arguments are in favor of the preferential use of the first variant for the screening of natural products with antibacterial activity. The hole plate method is the only suitable diffusion technique for testing aqueous suspensions of plant ethanol extracts [16]. In this method, the presence of suspended particulate matter in the sample being tested is much less likely to interfere with the diffusion of the antimicrobial substance into the agar than in the filter paper disc.

A polar compound would not be influenced by the hydroxyls on the surface of the paper and would diffuse easily. Thus, in this case, well agar diffusion method is more convenient than the disc variant. This theory explains at least in part, the higher sensitivity detected by well agar diffusion method. Also, the fact that the well variant yielded larger growth inhibition zones might be related to natural products transport by carrier compounds such as DMSO, for it is expected that they diffuse easily across the medium.

Based on the preliminary phytochemical analysis of leaves gave the presence of major compounds such as alkaloids, phenolic acids and Saponins which were known to have antibacterial activity tempted us to perform the thin layer chromatography. We have tried various solvent systems by trial and error methods. Finally, the mobile phases in table-1 gave good resolution in separation of compounds. We have obtained the four compounds even on the alteration in the mobile phase, finally the solvent systems such as Toluene: Ethyl acetate: Formic acid separated compounds at 0.6, 0.88, 0.94 and 0.98. The mobile phase with Toluene: Ethyl acetate (1:1) resolute compounds at 0.63, 0.86, 0.90 and 0.96. Finally the solvent system Hexane: Toluene: Ethyl acetate: Formic acid (2: 5: 2.5: 0.5) also gave four spots at R_f values of 0.41, 0.6, 0.81 and 0.89 which gave variation of compounds on separation which can be seen in fig. 1

Based on the chromatogram obtained from methanolic extract of leaves, in our studies which was tempted us to identify the active component led for activity by bioautography technique which was simple, less expensive and easy to perform. One of the mobile phase which were given in table-4 were tried for identification of active fractions

identification by bioautography were found at 0.63, 0.86 and 0.6 and 0.88 against most sensitive organisms such as *K.pneumoniae* and *P.putida*.

Finally, it is essential to note that the isolation, identification and purification of compounds led for activities by bioautography should identified scientifically by sophisticated techniques like NMR, column chromatography, HPTLC for development of drugs which substitute synthetic drugs which impose the adverse effects. Such research should lead to development of herbal antibiotic era.

Conclusion

The processing of the plant part used in the study was not comparable to the traditional approach when the ancients used water for extraction where we have used methanol for extraction in the sense it is not an exact replication of traditional knowledge. All the same given that methanol extract was more effective than water extract. The antibacterial activity of *X. strumarium* was reported for first time. Hence, finally the extract should be isolated, identified characterized and purification of compounds by scientific evidence with novel techniques for evaluation.

Table 1: Preliminary Phytochemical Analysis of *Xanthium strumarium*

Alkaloids	Present (++)
Carbohydrates	Absent (-)
Glycosides	Present (+)
Saponins	Present (+)
Phytosterols	Present (+)
Fixed oils	Present (+)
Resins	Absent (-)
Phenols	Present (++)
Tannins	Absent (-)
Flavonoids	Absent (-)
Proteins and Amino acids	Absent (-)
Diterpenes	Present (++)

High Amount – (++); Moderate – (+); Absent – (-)

Table 2: Antibacterial activity of methanolic extract (Leaf) of *Xanthium strumarium*

S. No	Strain	Methanolic extract (mm)		Amikacin (5µg/ml)
		50 mg/ml	100 mg/ml	
1	*P.putida ATCC 12842*	15	15	22
2	*S.epidermidis ATCC 12228*	10	13	12
3	*B.cereus ATCC 12842*	10	11	12
4	*P.aeruginosa ATCC 27853*	11	13	16
5	*S.typhimurium ATCC 23564*	11	13	16
6	*K.pneumoniae NICM 2719*	15	15	14
7	*P.vulgaris NCTC 8313*	11	12	15
8	*B.subtilis ATCC 6633*	15	12	15

Table 3: TLC Profile of *Xanthium strumarium* Leaf Extract

S. No	Plant Part	Mobile Phase	R_F Values
1	Leaf	Toluene: Ethyl Acetate: Formic Acid (5:4:1)	0.6, 0.88,0.94,0.98
2	Leaf	Toluene: Ethyl Acetate (1:1)	0.63,0.86,0.90,0.96
3	Leaf	Hexane:Toluene:Ethyl Acetate: Formic Acid (2:5:2.5:0.5)	0.41,0.6,0.81,0.89

Table 4: TLC bio-autography of Methanolic extract of *X. strumarium*

S. No	Plant part	Solvent for extraction	Solvent system	R_F Values	Strain
1	Leaf	Methanol	Toluene: Ethyl acetate: Formic acid(5:4:1)	0.6 and 0.88	*K.pneumoniae P.putida*
2	Leaf	Methanol	Toluene: Ethyl acetate (1:1)	0.63 and 0.86	*P.putida K. pneumoniae*

Fig.1: TLC of the *Xanthium strumarium* leaf methanolic extract

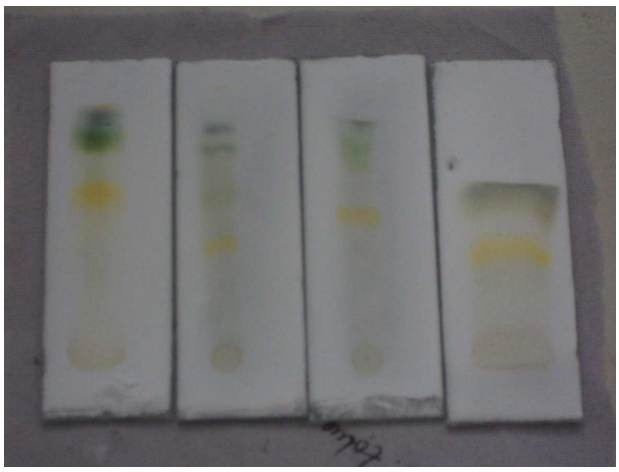


PLATE 1

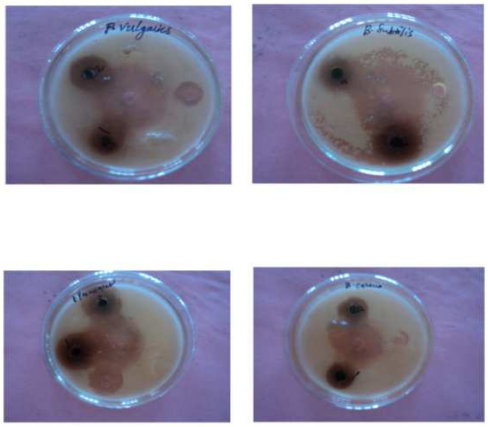
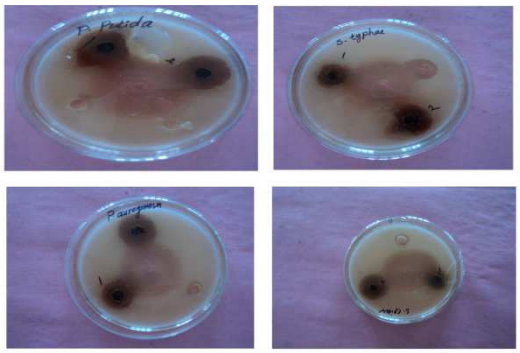


PLATE 2



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