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Research Article

IN VITRO SCREENING OF ANTIMICROBIAL ACTIVITY OF *WRIGHTIA TINCTORIA* (ROXB.) R. BR.

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ABSTRACT

An *in-vitro* antimicrobial study was done using methanolic and petroleum ether extracts from the leaves of *Wrightia tinctoria* (Roxb.) R.Br. Bauer-Kirby (disc diffusion) and broth dilution methods were employed for the assessment of antimicrobial activity against 14 microorganisms. Methanolic extract of *Wrightia tinctoria* leaves showed significant antimicrobial activity against *Cryptococcus neoformans* (36.0mm), *Staphylococcus aureus* (27.2mm), *Candida albicans* (25.0mm), *S.epidermidis* (23.2mm) and *Bacillus subtilis* (20.2mm), whereas petroleum ether leaves extract showed significant antimicrobial activity against *S.aureus* (25.0mm), *C.neoformans* (21.8mm), *S.epidermidis* (18.5mm) and *C.albicans* (16.0mm) were observed. According to broth dilution method, the methanolic extract of plant material showed the MIC values against *C.neoformans* (256µg/ml), *S.aureus* and *C.albicans* (512µg/ml) respectively. Whereas, the petroleum ether extract of *Wrightia tinctoria* showed the MIC values against *S.aureus* and *C.neoformans* (512µg/ml) and *S.epidermidis* and *C.albicans* (1,024µg/ml) with a significant inhibitory activity. The present result revealed that methanolic and petroleum ether extracts of *Wrightia tinctoria* possesses both antibacterial and antifungal activity.

Keywords: *Wrightia tinctoria*, MIC, Antibacterial activity and Antifungal activity

INTRODUCTION

Plants have been used as medicinal agents from the earliest days of man's existence. Effective early remedies were subsequently recorded and documented, leading to the early herbals. Pharamacognosy - the knowledge of drug grew from these records to provide a disciplined, scientific description of natural materials used in medicine [1]. In the recent years, there has been a renewed interest on medicinal plants and plant based drugs as it gives a holistic and harmonious balance of physical, biological and mental process through systems of medicine like Ayurvedha, Unani, Siddha and Homeopathy, due the emergence of multi-drug resistance microorganisms [2]. In particular, the antimicrobial activity of plant extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [3]. Antimicrobials of plant origin are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with scientific antimicrobials [4]. Alternatively tremendous development in the field of modern medicine, plants still rank first in modern as well as traditional medicine throughout the world [5]. Hence, the plant kingdom is being screened for the newer and effective chemotherapeutic agents. Higher plants can serve both as potential antimicrobial crude drugs as well as a source of new anti-infective agents [6].

Wrightia tinctoria (Roxb.) R.Br. belongs to family Apocynaceae (Oleander family) is a small, deciduous tree with a light gray, scaly, smooth bark, generally up to 1.8m tall and often under 60cm girth, sometimes up to 7.5m high, native to India and Burma [7]. *Wrightia* is named after a Scottish physician and botanist called William Wright (1740-1827), *Wrightia tinctoria* is commonly called as "Sweet Indrajao", "Pala Indigo plant", "Dyer's Oleander" and locally as "Paalai". The leaves of this tree yield a blue dye called "Pala Indigo". The five flavonoid compounds indigotin, indirubin, tryptanthrin, isatin and rutin were isolated and identified from the leaves [8]. A stable serine protease, Wrightin has been isolated from the latex of the plant *W.tinctoria* [9]. The characterization of ligno-cellulosic seed fibre from *W.tinctoria* has been carried out [10]. Different extracts of leaf parts of *W.tinctoria* and fruit powder of *Morinda Citrifolia* have been studied against replication of HIV-1 [11]. The juice of the tender leaves is used efficaciously in jaundice. Crushed fresh leaves when filled in the cavity of the decayed tooth relieve tooth ache [7]. The leaves are applied as a poultice for mumps and herpes. The bark of *W.tinctoria* is considered for antidiarrhoeal, aphrodisiac, anthelmintic, febrifuge, stomachic, tonic and dog bite [12]. It is employed in seminal weakness and flatulence [13] also used in piles and skin diseases [13]. In folk medicine, the dried and powdered roots of *Wrightia* along with *Phyllanthus amarus* (Keezhanelli) and *Vitex negundo* (Nochi) is mixed with milk and orally administered to women for improving fertility. The bark and seeds are effective against psoriasis, wound healing, non-specific dermatitis, anti-dysentric, anti-haemorrhagic, and hepatoprotective activity, anti-inflammatory and anti-dandruff properties. The leaves are useful in odontalgia, vitiated conditions of vata and hypertension.

Over the past 20 years, there has been a lot of investigation on the plants as a source of new antimicrobial agents. Recent studies have shown that several alcoholic extracts of various traditional medicinal plants exhibit antibacterial activity [14]. The present study has been extensively investigated antimicrobial activity of *Wrightia tinctoria* (Roxb.) R.Br. leaves methanol and petroleum ether extracts on selected organisms.

MATERIALS AND METHODS

Collection of plant materials

Fresh plant leaves of *Wrightia tinctoria* (Roxb.) R.Br. were collected from the natural habitat of Ponmudi hills, Kerala. The plant species was identified by Dr.Santhosh Kumar, scientist, Tropical Botanic Garden and Research Institute, Thiruvananthapuram, Kerala.

Extraction of plant materials

The dried leaf material was pulverized into fine powder using a griender (mixer). About 50gms of powdered material was extracted by cold maceration method successively with 300 ml of each of the following solvents *viz*., methanol and petroleum ether and kept for 7 days at room temperature. The extracts obtained with each solvent were filtered through Whatmann filter paper No.1 and the respected solvents were evaporated (40˚C) with help of heating mantle. The sticky greenish-brown substances were obtained and stored in refrigerator and were suspended in dimethyl sulphoxide (DMSO) for prior to use.

Antimicrobial Screening

Source of microbial strains

Strains of human pathogenic microorganisms used in this study are as follows, three gram positive bacteria, *viz*., *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 435) and *Bacillus subtilis* (MTCC 121) and nine gram negative bacteria, *viz*., *Escherichia coli* (MTCC 443), *Klebsiella pneumoniae* (MTCC 432), *Proteus vulgaris* (MTCC 744), *Proteus mirabilis* (MTCC 1429), *Salmonella paratyphi* A (MTCC 735), *Salmonella paratyphi* B (clinical isolate), *Salmonella typhimurium* (MTCC 98), *Shigella flexneri* (MTCC 1457), and *Vibrio parahaemolyticus* (MTCC 451); two fungus,

Candida albicans (MTCC 183) and *Cryptococcus neoformans* (clinical isolate). The microorganisms were originally obtained from Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India. Cultures were maintained as nutrient agar slants in screw-capped bottles and stored at 4˚C. All cultures were checked for viability and purity by regular plating.

Minimum inhibitory concentration (MIC) - Disc diffusion method

The antimicrobial activities of the successive leaf extracts of methanol and petroleum ether were tested by disc diffusion method [15]. The culture plates were prepared by pouring 20 ml of sterile Hi-sensitivity (Himedia- M486) agar. The depth of the medium was approximately 4mm. 3-4 similar colonies of pure cultures were inoculated with Tryptone soy broth (Himedia - M 323), further, it was incubated at 37˚C for 2-8 hours and inoculum size was adjusted to yield uniform suspension containing 10^5-10^6 cells/ml (McFarland's standard). The agar surfaces of the plates were swabbed with test culture in three directions, turning the plates at 60˚C angle between each swabbing. Confluent growth is desirable for accurate results. The sterile discs (6mm - Himedia) were used for the loading crude plant leaves extracts (methanol and petroleum ether). Five different concentrations were prepared (250µg, 500µg, 750µg, 1000µg and 1250µg) and loaded in appropriate discs. The impregnated discs were incubated at 37˚C for an hour. The dried discs were placed over the surface of swabbed medium with equal distance to avoid the overlapping of the zone of inhibition. Then press the discs lightly on the surface of the medium. Allowed the plates to stand at refrigerator for 30minutes (Pre-diffusion time). The plates were incubated at 37˚C for 16-18 hours during which the activity was evidenced by the presence of zone of inhibition surrounding the discs. Each experiment was done in triplicate. A panel of antibiotics was used against each microbial strain and which antibiotic given sensitive with particular organism used as control.

Minimum Inhibitory concentration (MIC) - Broth dilution method

Tube dilution method was used to determine the minimum inhibitory concentration (MIC) of the extracts in Muller Hinton broth (Himedia-M 391) and Sabouraud Dextrose Broth (Himedia-M 033) as specified by National Committee for Clinical Laboratory Standard (NCCLS, 1998). 10ml of each broth was dispensed into separate test tube and was sterilized at 121˚C for 15 minutes and then allowed to cool. Two-fold serial dilutions of the extracts in the broth were made from the stock concentration of the extracts to obtain 8µg/ml to 4096µg/ml for methanol and petroleum ether extracts. 0.1ml of the standardized inoculums of the microbes was inoculated into the different concentration of the extracts in the broth. The test tubes of the broth were incubated at 37˚C for 24hrs and 30˚C for 1-7 days for bacteria and fungi respectively and observed for turbidity. The lowest concentration which showed no turbidity in the test tube was recorded as the MIC.

Determination of Activity Index

The activity index of the crude plant extract was calculated as;

$$\text{Activity Index (AI)} = \frac{\text{Zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic Drug}}$$

RESULT

The antimicrobial activity of the extracts (methanolic and petroleum ether) at different concentrations were screened by the disc diffusion method and the mean value of zone of inhibition was assessed in millimetre diameter. The results are given in the tables-1, 2 and figures-1, 2. The minimum inhibitory concentration (MIC) was determined by the broth dilution method and the results are given in table-3.

Table 1: Antimicrobial activity of leaves extract of *Wrightia tinctoria* (Roxb.) R.Br

S.No.	Name of the Organism	Zone of Inhibition in mm			Standard Antibiotic µg/disc	Zone in mm	Activity Index (AI)
		Methanolic Leaves Extract					
		Min.	Max.	Mean ±SEM			
1.	*Staphylococcus aureus*	24	30	27.2 ± 0.91	Amoxycillin/ Clavulanic acid (30)	30	0.90
2.	*Staphylococcus epidermidis*	18	25	23.2 ± 1.27	Amoxycillin/ Clavulanic acid (30)	30	0.77
3.	*Bacillus subtilis*	15	25	20.2 ± 1.59	Ciprofloxacin (5)	32	0.63
4.	*Escherichia coli*	-	-	-	Ciprofloxacin (5)	30	0.00
5.	*Klebsiella pneumoniae*	-	-	-	Chloramphenicol (30)	30	0.00
6.	*Proteus vulgaris*	-	13	13.0 ± 3.67	Co-trimoxazole (25)	26	0.50
7.	*Proteus mirabilis*	-	-	-	Lomefloxacin (10)	25	0.00
8.	*Salmonella paratyphi A*	-		-	Chloramphenicol (30)	27	0.00
9.	*Salmonella paratyphi B*	-	-	-	Ciprofloxacin (5)	28	0.00
10.	*Salmonella typhimurium*	-	-	-	Ciprofloxacin (5)	32	0.00
11.	*Shigella flexneri*	-	10	9.5 ± 3.29	Lomefloxacin (10)	36	0.26
12.	*Vibrio parahaemolyticus*	-	-	-	Ciprofloxacin (5)	29	0.00
13.	*Candida albicans*	20	30	25.0 ± 1.49	Amphotericin (20)	15	1.66
14.	*Cryptococcus neoformans*	22	42	36.0 ± 3.57	Ketoconazole (10)	35	1.02

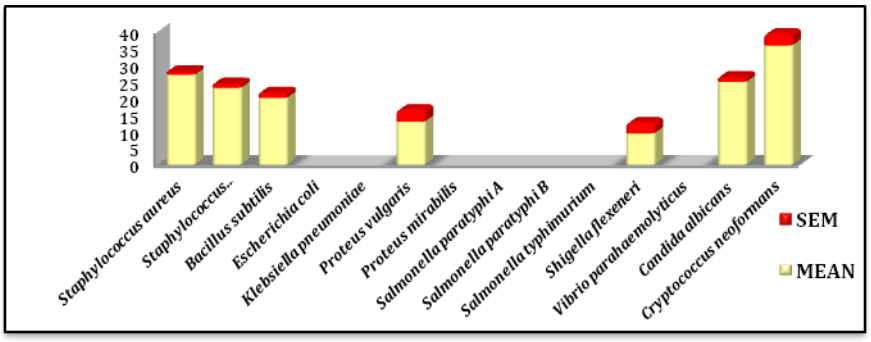

Figure 1:Mean and Standard Error of Antimicrobial Activity of Methanolic Extract of *Wrightia tinctoria* Roxb.R.Br.

Table 2: Antimicrobial activity of leaves extract of *Wrightia tinctoria* (Roxb.) R.Br.

S.No.	Name of the Organism	Zone of Inhibition in mm					
		Methanolic Leaves Extract			Standard Antibiotic µg/disc	Zone in mm	Activity Index (AI)
		Min.	Max.	Mean ±SEM			
1.	*Staphylococcus aureus*	21	27	25.0 ± 1.01	Amoxycillin/ Clavulanic acid (30)	30	0.83
2.	*Staphylococcus epidermidis*	-	20	18.5 ± 3.84	Amoxycillin/ Clavulanic acid (30)	30	0.61
3.	*Bacillus subtilis*	-	16	13.8 ± 3.07	Ciprofloxacin (5)	32	0.43
4.	*Escherichia coli*	-	-	-	Ciprofloxacin (5)	30	0.00
5.	*Klebsiella pneumoniae*	-	-	-	Chloramphenicol (30)	30	0.00
6.	*Proteus vulgaris*	-	-	-	Co-trimoxazole (25)	26	0.00
7.	*Proteus mirabilis*	-	-	-	Lomefloxacin (10)	25	0.00
8.	*Salmonella paratyphi A*	-	-	-	Chloramphenicol (30)	27	0.00
9.	*Salmonella paratyphi B*	-	-	-	Ciprofloxacin (5)	28	0.00
10.	*Salmonella typhimurium*	-	10	10.0 ± 4.00	Ciprofloxacin (5)	32	0.31
11.	*Shigella flexneri*	-	-	-	Lomefloxacin (10)	36	0.00
12.	*Vibrio parahaemolyticus*	-	-	-	Ciprofloxacin (5)	29	0.00
13.	*Candida albicans*	-	16	16.0 ± 4.52	Amphotericin (20)	15	1.06
14.	*Cryptococcus neoformans*	11	28	21.8 ± 3.01	Ketoconazole (10)	35	0.62

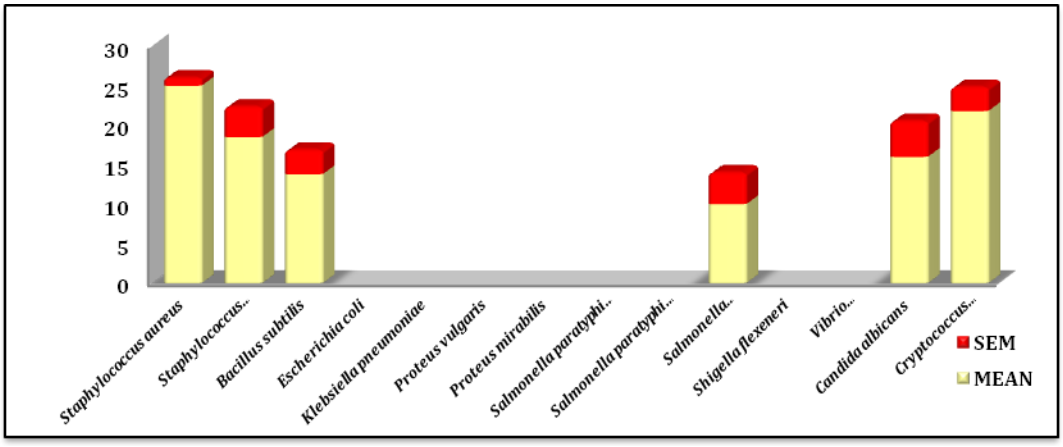

Figure 2:Mean and Standard Error of Antimicrobial Activity of Petroleum Ether Extract of *Wrightia tinctoria* Roxb.R.Br.

Table 3: Minimum Inhibitory Concentration of leaves extract of *Wrightia tinctoria* (Roxb.) R.Br.

S.No.	Name of the organism	Concentration of extracts (in µg/ml)											
		4096	2048	1024	512	256	128	64	32	16	8	Control	MIC in (µg /ml)
	Methanol extract												
1	*Staphylococcus aureus*	-	-	-	-	+	+	+	+	+	+	+	512
2	*Staphylococcus epidermidis*	-	-	-	+	+	+	+	+	+	+	+	1024
3	*Bacillus subtilis*	-	-	+	+	+	+	+	+	+	+	+	2048
4	*Candida albicans*	-	-	-	-	+	+	+	+	+	+	+	512
5	*Cryptococcus neoformans*	-	-	-	-	-	+	+	+	+	+	+	256
	Petroleum ether extract												
1	*Staphylococcus aureus*	-	-	-	-	+	+	+	+	+	+	+	512
2	*Staphylococcus epidermidis*	-	-	-	+	+	+	+	+	+	+	+	1024
3	*Candida albicans*	-	-	-	+	+	+	+	+	+	+	+	1024
4	*Cryptococcus neoformans*	-	-	-	-	+	+	+	+	+	+	+	512

The methanolic extract of *Wrightia tinctoria* leaves showed significant antimicrobial activity against *Cryptococcus neoformans* (36.0±3.57 mm, AI-1.02), *Staphylococcus aureus* (27.2±0.91 mm, AI-0.90), *Candida albicans* (25.0±1.49 mm, AI-1.66), *S.epidermidis* (23.2±1.27 mm, AI-0.77) and *Bacillus subtilis* (20.2±1.59 mm, AI-0.63) were documented. Methanolic extract exhibits significant antifungal activity when compared than the control antifungal agents (Ketaconazole and Amphotericin-B), methanolic extract showed good antibacterial activity and the result also nearer to the zones produced by the (Amoxycillin/Clavulanic acid and Ciprofloxacin) control anti-bacterial agents.

In disc diffusion method, the methanolic extract showed equal and more than 15mm mean zone of inhibition was documented against microorganisms were tested for minimum inhibitory concentration (MIC) by broth dilution technique. The result revealed that 256µg/ml was observed as MIC value against *Cryptococcus neoformans*, 512µg/ml was documented against *Staphylococcus aureus* and *Candida albicans*, 1,024µg/ml (*S.epidermidis*) and 2,048µg/ml (*Bacillus subtilis*) were observed. The result indicated that the methanolic extract possesses good inhibitory activity against both fungi and bacteria.

The petroleum ether extracts showed significant antimicrobial activity against *Staphylococcus aureus* (25.0±1.01 mm, AI-0.83), *Cryptococcus neoformans* (21.8±3.01 mm, AI-0.62), *S.epidermidis* (18.5±3.84 mm, AI-0.61) and *Candida albicans* (16.0±4.52 mm, AI-1.06) were documented. Petroleum ether extract exhibited significant antifungal activity against *C. albicans* when compared than the control antifungal agent (Amphotericin-B), it also showed good antibacterial activity and the result also so nearer to the zones produced by the (Amoxycillin/Clavulanic acid) control antibacterial agents.

In disc diffusion method, the petroleum ether extract showed equal and more than 15mm mean zone of inhibition was documented against microorganisms were tested for minimum inhibitory concentration (MIC) by broth dilution technique. The result revealed that 512µg/ml was observed as MIC value against *Staphylococcus aureus* and *Cryptococcus neoformans* and 1024µg/ml (*S.epidermidis* and *Candida albicans*) were also observed. The result indicated that the petroleum ether extract possesses significant inhibitory activity against both fungi and bacteria.

DISCUSSION

Recently, a number of antibiotics have lost their effectiveness due to the development of resistant strains of bacteria, which has primarily occurred through the expression of resistance genes [16, 17]. In addition to including resistance, antibiotics are sometimes associated with opposing effects such as hypersensitivity, immune-suppression and allergic reactions [18]. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases [19, 20]. The antimicrobial effect of *Wrightia tinctoria* was previously studied. Indeed, it is reported that the many research outcomes made with bark of *W.tinctoria* [14, 21, 22]. The aqueous extract of *W.tinctoria* (leaves) are showed significant inhibitory activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa* when compared as methanolic extract [7]. Contraversly, the methanolic extract of *W. tinctoria* (leaves) had significant inhibitory activity against most of the organism when compared as aqueous extract [23]. These results are in accordance to results obtained in the present study exhibited both antibacterial and antifungal activity was observed against 50% of the organisms tested. The methanolic extract of *W. tinctoria* leaves showed excellent inhibitory activity against *Cryptococcus neoformans* (36.0mm), *S.aureus* (27.2mm), *Candida albicans* (25.0mm) and *S.epidermidis* (23.2mm) were documented. Significance of the present result interestingly noted that methanolic extract of *W.tinctoria* possess significant antifungal activity when compared as control antifungal agents (Ketoconazole-35mm and Amphotericin-B-15mm). The result of broth dilution also showed inhibitory effects against *C.neoformans* (250µg/ml), S.*aureus* and *C.albicans* (512µg/ml). Similarly petroleum ether extract of *W.tinctoria* (leaves) showed antimicrobial activity against *S.aureus* (25.0mm), *C.neoformans* (21.8mm), *S.epidermidis* (18.5mm) and *C.albicans* (16.00m) were documented. The results of MIC values of petroleum ether extract showed less effectiveness when compared as methanolic extract. These findings and the results obtained in our study clearly confirm the effectiveness of *W.tinctoria* leaves extract on inhibition of microbial activity. The methanolic and petroleum ether extracts of woody stem of *W.tinctoria* and results shows broad spectrum antimicrobial activity most of organisms tested and who reports moderate antimicrobial activity of petroleum ether extracts [6]. There are many reports of antimicrobial activity of *W.tinctoria* leaves extract showing that methanolic and petroleum ether extracts are found to have significant to moderate antimicrobial activity against various organisms [7, 14, 23]. The antimicrobial activity of *W.tinctoria* leaves extract might be related to the action of its antibiotic compounds or to the presence of metabolic toxins. Inhibitory activity of *W.tinctoria* may be associated with the presence of alkaloids, steroids, saponins and flavonoids [7]. Another research outcome also reports the steroids, triterpenoids, flavonoids and glycosides in this plant [24]. This suggests that these components may also provide antimicrobial activity against certain microorganisms and provides a plausible explanation for the higher antimicrobial activity of these extracts. On the other hand, a few unknown minor components present have not been elucidated in terms of their activity. Further studies then need to be done. In the nearest future, thorough investigation is needed to better ascertain the antimicrobial effect of these plant extracts.

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