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In-Vitro ANTIMICROBIAL SYNERGISTIC AND ANTI-TB ACTIVITIES OF *PHYLLANTHUS ACIDUS* METHANOLIC EXTRACT

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Abstract

The objective of the present study was to evaluate the synergistic antimicrobial and anti-tubercular activities of *phyllanthus acidus* Methanolic extract. Antibacterial study was carried out by plate hole diffusion or agar well diffusion assay to determine the growth inhibition of bacteria. Antifungal activity was performed by the use of Saubouraud dextrose agar medium (SDA). The synergistic activity study was calculated by means of cup plate method (Kirbauy bauer technique) using two wells in a plate. The Methanolic plant extract of *Phyllanthus acidus* (500µg/ml) was used in combination with Oxytetracycline (500µg/ml). Anti-tubercular assay was performed using Micro plate Alamar Blue Assay (MABA) using the suspension of *Mycobacterium tuberculosis* H37Rv strain. The concentrations of plant extract were used are 1000 µg/ml, 500µg/ml, 250µg/ml, 125 µg/ml, 62.5µg/ml. The medicinal plant appear to have a broad antimicrobial activity spectrum, they could be useful in antiseptic and disinfectant formulation as well as in anti-tubercular activity. Among the various micro organisms, the Methanolic extract was more active against *Micrococcus flavum*. In antifungal activity the Methanolic extract shows positive results for all fungus. The anti-tubercular activity was compared with standard drug Rifampicin. The Methanolic extract was having more percentage inhibition when compared to other extracts.

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INTRODUCTION

Natural products especially medicinal plants have long been prescribed in traditional medicine for centuries for treating various diseases. The importance of herbs in the management of human ailments cannot be over-emphasized. Medicinal plants are a source of great economic value in the Indian subcontinent. Medicinal plants have been used for centuries as remedies for human diseases because they contain components of therapeutic values. The scientific studies available on a good number of medicinal plants indicates that promising phytochemical can be developed for many human health problems including diabetes, cancer and infectious diseases. The continued investigation into the secondary plant metabolites for anti-infective agents has gained importance because of the alarming increase in the rate of resistance of pathogenic microorganism to existing antibiotics. Therefore the need develop efficient, safe and inexpensive drugs from plant sources is of great importance[1].

Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country. India is rich in all the 3 levels of biodiversity, species diversity, genetic diversity and habitat diversity. In India thousands of species are known to have medicinal value and the use of different parts of several medicinal plants to cure specific ailments has been in vogue since ancient times[2]. The main aim to investigate the natural antimicrobial and anti-tubercular activities of *Phyllanthus acidus* extract. This observation stimulates the search for new antimicrobial and anti-tubercular agent and the naturally occurring compounds could be very valuable.

MATERIALS AND METHODS

Collection of Plant materials

Phyllanthus acidus leaves used for this study were obtained from (host plant) in Deviyakurichi, Salem district, TamilNadu, India. The plant leaves were identified by Botanical Survey India, Coimbatore and the voucher samples are kept in the BSI herbarium for reference **(BSI/SC/5/23/08-09/Tech-613).**

1 Phytochemical Studies[3]:-

The preliminary phytochemical screening of *phyllanthus acidus* was carried out for the decoction of various phytoconstituents using standard procedure of Harbone. The following solvents were used for the study, Chloroform, Ethyl acetate, Methanol, Ethanol and Water. The Methanolic extract was found to contain more constituent. The Preliminary phytochemical screening of methanol extract reveals the presence of Alkaloids, Flavonoids, Tannins, Glycosides and Triterpenoids.

Preparation of Plant Extract[4]:

The powdered plant materials (10 gm) were extracted with 100 ml of methanol of 1hr on an ultrasonic bath. The extract was filtered through Whatmann filter paper, the filtrate was evaporated in vacuum at 45^0C. The extracts were prepared according to the polarity starting from n-hexane to methanol. The residue thus obtained was thus dried in vacuum desiccator to remove the final traces of solvents completely.

Table 1
Anti-bacterial study of Methanolic extracts of *phyllanthus acidus*.

Microorganisms	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.5 mg/ml	Oxytetracycline (1mg/ml)
Bacillus lichenformis (NCIM 2468)	19	14	10	08	0	26
Brevibacterium leuteum (ATCC 15830)	16	14	07	0	0	24
Escherichiae coli (ATCC 15830)	17	12	09	07	0	24
Flavobacterium devorans (NCIM 2581)	17	10	0	0	0	22
Klebsiella pneumoniae (ATCC 11229)	17	10	07	0	0	22
Micrococcus flavum (NCIM 2984)	22	16	07	11	0	20
Micrococcus leuteum (NCIM 2984)	16	11	08	06	0	20
Proteus mirabilis (NCIM 8268)	17	14	09	07	0	19
Rhodococcus terrae (NCIM 5126)	18	12	07	08	0	25
Salmonella typhi	19	15	09	08	0	28
Shigella boydi (ATCC 8700)	16	12	07	0	0	23
Shigella flexneri (NCIM 4924)	12	0	0	0	0	25
Shigella sonei (ATCC 29930)	16	10	08	06	0	22
Staphylococcus faecalis(ATCC 8043)	16	12	8	0	0	28
Staphylococcus aureus	19	13	10	06	0	25

Table 2
Anti-Fungal Study of Methanolic Extract of *Phyllanthus acidus*.

S.No	Micoorganisms	1000 µg/ml	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml	A*
01.	*Aspergillus niger (NCIM 1207)*	18	10	08	0	0	20
02.	*Candida albicans (NCIM 3484)*	20	14	11	09	0	24
03.	*Monilinia fruticola (NCIM 1011)*	15	12	07	0	0	22
04.	*Auricularia polytricha (NCIM 1303)*	14	10	0	0	0	23
05.	*Chaetomella raphigera (NCIM 1231)*	15	11	07	0	0	25
06.	*Arthrobotrys oligospora (NCIM 11246)*	18	12	08	06	0	22

A* - Standard Amphotericin-B (1000µg/ml)

Preparation of plant extracts:[4]

The root was chopped to small pieces and dried in shade. The dried root was powdered and a weighed quantity of the powder (650 g) was passed through sieve number 20 and subjected to hot solvent extraction in a soxhlet apparatus using aqueous MeOH (50:50), at a temperature range of 60-70˚C. Before and after every extraction the marc was completely dried and weighed. The extract was concentrated to dryness at 40˚C under reduced pressure in a rotary vacuum evaporator. The aqueous MeOH (50:50) extract yielded a brown semi-solid, weighing 78.0g (12.0%) and the extract was preserved in a refrigerator for its usage.

Table 3
Synergistic Activity of Methanolic Extract of *Phyllanthus acidus*.

S. No	Micro-organisms	Zone of inhibition (mm)
1.	*Shigella soneii (ATCC 29930)*	43
2.	*Streptococcus faecalis (ATCC 8043)*	44
3.	*Bacillus licheniformis (NCIM 2468)*	47
4.	*Klebsiella pneumoniae (ATCC 11229)*	46
5.	*Micrococcus leuteus (ATCC 9341)*	42
6.	*Flavobacterium devorans (NCIM 2581)*	43
7.	*Shigella boydii (ATCC 8700)*	42
8.	*Proteus mirabilis (NCIM 8268)*	45
9.	*Salmonella typhi*	48
10.	*Escherichia coli (ATCC 15830)*	46
11.	*Shigella flexneri (NCIM 4924)*	45
12.	*Micrococcus flavum (NCIM 2984)*	49
13.	*Brevibacterium leuteum (ATCC 15830)*	48

Table 4
MIC of *phyllanthus acidus* against *Mycobacterium tuberculosis*

S. No	Extract Fractions	MIC (µg/ml)	% Inhibition of concentrations
1.	Chloroform Extract	>50	22
2.	Ethyl acetate Extract	>50	17
3.	Methanol Extract	>50	36
4.	Aqueous Extract	>50	13
	Rifampicin	0.09	--

Micro-organisms

The test micro-organisms used are *Shigella sonei* (ATCC 29930), *Escherichiae coli* (ATCC 11229), *Streptococcus faecalis* (ATCC 8043), *Shigella boydi* (ATCC 8700), *Rhodococcus terrae* (NCIM 5126), *Micrococcus flavum* (NCIM 2984), *Flavobacterium devorans* (NCIM 2581), *Proteus mirabilis* (NCIB 8268), *Brevibacterium leuteum* (ATCC 15830), *Bacillus lichenformis* (NCIM 2468), *Shigella dysentriae* (ATCC 13313), *Klebsiella pneumoniae* (ATCC 11229), *Micrococcus leuteus* (ATCC 9341), *Shigella flexneri* (NCIM 4924). antitubercular organism used is *Mycobacterium tuberculosis* H37Rv. The various Fungi used for Antifungal study are *Aspergillus niger* (NCIM 1207), *Candida albicans* (NCIM 3484), *Monilinia fruticola* (NCIM 1011), *Auricularia polytricha* (NCIM 3484), *Chaetomella raphigera* (NCIM 1231), *Arthrobotrys oligospora* (NCIM 1246).

Standard Antibiotics used are Oxytetracycline, Kanamycin, Amphotericin-B, Rifampicin.

Preparation of 24 hours pure culture[5]

A loop full of each microorganism was suspended in about 10ml of physiological saline in a Roux bottle. Each of these was streaked on to the appropriate culture slants and was incubated at 37ºC for 24 hours except for *Candida albicans* which was incubated at 25ºC for 24-48 hours.

Fig : 1a and 1b shows the antibacterial activity of Methanolic extract of *Phyllanthus acidus* against *Micrococcus flavum.*

Fig 1a

Fig 1b

Fig: 2a and 2b Shows the Antifungal Activity of Methanolic Extract of *Phyllanthus acidus* against *Candida albicans.*

Fig 2a

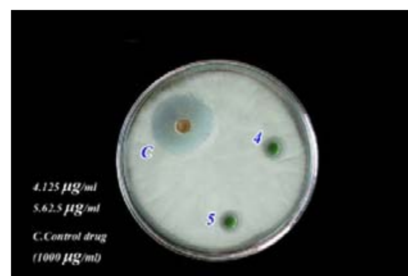

Fig 2b

Standardization of micro-organisms

Each of the 24 hours old pure culture was suspended in a Roux bottle containing 5 ml of physio-logical saline. Each suspension of micro-organisms was standardized to 25% transmittance at 560 nm using a Ultraviolet (UV)- visible spectrophotometer.

Antimicrobial study[6,7]

Antibacterial study (Plate hole diffusion method)

Antibacterial study (Plate hole diffusion or agar well diffusion) assay was used to determine the growth inhibition of bacteria by plant extracts. Bacteria were maintained at 4^0C on nutrient agar plate before use. Nutrient agar medium was prepared and each universals containing 20 ml was poured. The universals with the broth were inoculated with different bacterial species and

incubated at 37^0C for 24 hrs. A total of 25 ml of Molten Hinton (MH) agar was poured into sterile universals. Each universal was inoculated with 0.2 ml of different bacterial species mixed well with the MH into sterile Petri dishes and allow set. A well was prepared in the plates with the help of a cork- borer (6 mm) four holes per plates were made into the set agar containing the bacterial culture. A total of 0.2 ml of plant extract was poured in to the wells with concentration 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml. For each bacterial strain controls were maintained where pure solvents, instead of extract. The plates were incubated overnight at 37^0C. The results were obtained by measuring the diameter of the zone of inhibition. The result was compared with standard antibiotic Oxytetracycline (1000 µg/ml).

Antifungal activity

Saubouraud dextrose agar medium (SDA) was prepared and 25 ml of each was poured in to sterile universals. The universals with the broth were inoculated with different species of fungus and incubated at 28^0C overnight. A total of 25 ml of medium was poured into each sterile universal. Each universal was inoculated with 200 µl of different fungal species spread well and allows to set. Using a sterile cork borer 6 mm diameter, four holes per plate were made into the set medium containing fungal culture. A total of 0.2 ml of plant extracts were poured into the wells and one containing distilled water. The plates were incubated overnight for 36 to 48 hrs and the diameter of the zone of inhibition was then recorded if greater than 6 mm.

Synergistic activity study[8]

The synergistic activity study was calculated by combining with the standard antibiotics Oxytetracycline by means of cup plate method (Kirbauy bauer technique) using two wells in a plate. The Methanolic plant extract was of *Phyllanthus acidus* (500 µg/ml) was used in combination with Oxytetracycline (500 µg/ml). The distance between the two wells was maintained as standard of about 0.8 cm then incubated at 37^0C for 24 hrs and the diameter of the zone of inhibition was measured at second data.

Anti-tubercular assay[9]

Anti-tubercular assay was performed using Micro plate Alamar Blue Assay (MABA). Suspension of *Mycobacterium tuberculosis* H37Rv strain was prepared at a concentration of 105 cells/ml. Samples were dissolved in Dimethyl Sulphoxide (DMSO) and subsequent dilutions were performed in 0.1 ml of 7H9 medium in the micro plate together with the plant extract and its fractions (concentration 0.78 – 100 µg/ml). The plates were incubated at 37^0C for 7 days. At day 7 of incubation, 20 µl of Alamar blue solution were added to the control well. If the dye turned pink, indicating bacterial growth, the dye was then added to all remaining wells in the plate. The results were read on the following day and minimum inhibitory concentration (MIC) values of the extract and fractions were calculated. Rifampicin was used as positive control.

RESULTS AND DISCUSSION

From the results of antibacterial screening 100% of Methanolic extract were active in concentration of

1000 µg/ml, 93% active in concentration of 500 µg/ml, 87.5 % active in concentration of 250 µg/ml, 50% active in concentration of 125 µg/ml and no activity in lowest test concentration of 62.5 µg/ml. Antibacterial activity were showed in Table 1, The results of the antifungal study were reported in Table 2. The results of synergistic activity study showed that the Methanolic extract of the plant had good synergistic activity when combined with the standard antibiotic Oxytetracycline. The results of the synergistic study were reported in Table 3. The Methanol and chloroform extracts for the evaluation of the MIC to *Mycobacterium tuberculosis* H_{37} Rv with micro plate technique using Alamar blue test showed percentage of inhibition 36 and 22% at respectively with comparing standard drug Rifampicin. The percentage of inhibition ethyl acetate and aqueous extracts were found to be less active 17 and 13% respectively. So we concluded Methanolic extract was having more percentage inhibition when compared to other extracts. The result was shown in Table4.

The known antimicrobial mechanisms associated to flavonoids may explain the antimicrobial potency of these compounds from the crude extract. Under this study the extract capability to penetrate the cell walls with hydrophobic and hydrophilic environment. Plant showing significant activity may be due to the presence of alkaloids, flavonoids, tannins and polyphenols. Since the medicinal plant appear to have a broad antimicrobial activity spectrum, they could be useful in antiseptic and disinfectant formulation as well as in anti-tubercular activity. The Alamar blue used in MABA before assaying for mycobacterial activity in plant extracts did not interfere with the growth controls. The methods described here could be useful in determining the anti-tubercular activity of natural products because these assays require smaller volumes and can be performed faster than other methods such as Bactec 460 to expose mycobacteria to the anti-tuberculosis natural products and a solid medium to determine the number of CFU, which takes 21 days to complete. The new method requires just 7 days producing results (6 days to observe full growth in the corresponding controls and 1 day to develop the remaining wells). Furthermore, this method requires only 200 µl per well to perform the entire assay, where as others use 9-10 ml of solid medium.

CONCLUSION

Among the various microorganisms, the Methanolic extract was more active against *Micrococcus flavum* and *Candida albicans.* This result suggests the presence of either good antimicrobial activity or high concentration of an active principle in the extract. This antimicrobial activity would support the folk therapy of infections. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

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