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Anti-Dermatophytic Activity Of Piper Betle Linn. (Leaf Stalk)

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Abstract: ***In recent years, there has been increasing interest worldwide in the use of alternative/herbal medicine for the prevention and treatment of fungal diseases. Currently, however, quality-related problems (lack of consistency, safety, and efficacy) seem to be overshadowing the potential genuine benefits of various herbal products for the treatment of fungal diseases. Extracts obtained from many plants have recently gained a great popularity and scientific interest. Since the middle ages, natural plant preparations have been widely used for treatment of fungal diseases. Treatment of the fungal pathogen is becoming increasingly difficult due to antifungal drug resistance, especially with fluconazole, which is a commonly used azole. This paper presents the in vitro activity of medicinal plant piper betle (Leaf stalk) methanolic extracts and their major compounds against dermatophytes and also a compilation of updated information on medicinal plant extracts with antifungal and antibacterial properties.***

Keywords: Antidermatophytic activity, piper betle (Leaf stalk), Methanolic extracts.

I. INTRODUCTION

Piper betle Linn. (Local name 'Pan') belongs to family *piperaceae*, a dioecious, perennial creeper, climbing by many short adventitious rootlets, widely cultivated in hotter and damper parts of the country is wide spread in damp forests and is cultivation in India and other countries in South East Asia, such as Vietnam and China. In Ayurveda the leaf of P. betle are used as acrid, healing, tonic, carminative, stomachic, anthelmintic, aphrodisiac, laxative, bronchitis, elephantiasis of the leg and to improve appetite. But it should not be taken in eye diseases, leprosy, poisoning thirst, alcoholism and asthama. In Unani system of medicine leaves are used to improve taste, appetite, tonic to the brain, in heart and liver diseases, strengthens the teeth and clear the throat. The juice of leaves is dropped into the eye in night blindness (B.D. Basu). The present study involves leaf stalk of *Piper betle L.* for evaluating antidermatophytic activity.

II. MATERIALS AND METHODS

PLANT COLLECTION AND IDENTIFICATION

The *P.betle* plant material was collected from Kolkata (West Bengal). The leaf stalk studied was collected from plants grown in Kolkata, West Bengal. A voucher specimen has been deposited at the herbarious of Vikram University, Ujjain (M.P.). The taxonomic identification of the plant material was obtained from the authorities of the institute of environment management and plant sciences, Vikram University, Ujjain (M.P.) India.

EXTRACTION OF PLANT MATERIAL BY SOXHLET APPARATUS

The plant materials after drying were ground in grinding machine in the laboratory then 25kg of shade dried powder was weighed and extracted with 98% methanol in Soxhelt extractor for 70hrs. The methanol extracts of were concentrated under reduced pressure and preserved in refrigerator in airtight bottle for further use.

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PREPARATION OF EXTRACT DILUTION SERIES

EXTRACT STOCK SOLUTION

Dissolved 400gm of crude in 10ml DMSO with glass beads, vortex to homogenize and a two-fold serial dilution was prepared. As a precaution not to miss trace amounts of antifungals for preliminary screening, a relatively high conc. Of 0.62 to 40mg/ml of each extract was prepared for bioassays.

TEST MICROORGANISMS

Five fungal cultures *Trichophyton rubrum, Microsporum gypseums, Trichophyton tonsurans, Aspergillus flavus, Candida albicans,* and five bacterial cultures *Bacillus subtilis, Escherchia coli, Staphylococcus aureus, Psudomonas sps., andBrevibacillus sps*. were used in the present study[12]. All the tested strains were obtained from department of microbiology R.D.Gardi Hospital Ujjain M.P. Bacterial cultures were grown in nutrient broth (Hemedia, M002) at 37^0C and maintained on nutrient agar slants at 4^0C, fungal cultures were grown in potato dextrose agar slants at 4^0C.

MEDIA FOR INOCULATION

Sabouraud's Dextrose Agar medium (SDA)

Peptone	-	10.0g.
Dextrose	-	40.0g.
Agar	-	20.0
Cycloheximide	-	0.5g.
Chloramphenicol	-	1 x 250mg Capsule.
Distilled water	-	1000ml.
pH	-	5.6

Sabouraud's Dextrose Medium (SDB)

Peptone	-	10.0g.
Dextrose	-	40.0g.
Cycloheximide	-	0.5g.
Chloramphenicol	-	1 x 250mg Capsule.
Distilled water	-	1000ml.
pH	-	5.6

Potato Dextrose Agar (PDA)

Peeled potato	-	250g
Glucose	-	20.0g
Agar	-	20.0g
Distilled water	-	1000ml
pH	-	6.0- 6.5

Nutrient Broth

Peptone	-	10.0g
Beef extract	-	0.03g
Sodium chloride	-	0.05g
Distilled water	-	1000ml
pH	-	5.0

FUNGAL INOCULUM PREPARATION

The dermatophytes were grown on SDA for a week and the spores were collected in by flooding with 0.85% saline (Ghannom et al, 2004). After settling the large particles in the test tube, the supernatant was taken and counted the number of conidia using hemocytometer. A tn fold dilution was made 9ml of normal saline solution was taken in five test tubes. In first test tube, 1ml of spore suspension was poured into test tube under aseptic conditions. The solution of first test tube was homogenized and 1ml of this solution was transferred to second test tube containing 9ml of normal saline solution. This process was repeated upto5^{th} test tube. In each case sterilized pipette was used. From every test tube (for each dilution), 0.1ml suspension was transferred to sterilized SDA petri plates. Triplicates of each dilution were maintained. The SDA seeded petri plates were counted in hemocytometer. The average of 3 petri plates was taken in each case. The test inoculums were adjusted between 1.5x 10^5 spores/ml.

ANTIDERMATOPHYTIC ACTIVITY

DETERMIBATION OF MIC BY AGAR WELLDIFFUSION METHOD (MEGALDI S ET AL, 2004)

The assay was performed by agar well diffusion method. About 16 to 20ml of potato dextrose agar medium was poured in the sterilized petri dishes and allowed to solidify. Fungal lawn was prepared using 5 days old culture strain. The fungal strains were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 Mac Farland Standards (108 CFU/ml). 1ml of fungal strain was spread over the medium using sterilized glass spreader. Using flamed sterile borer, wells of 4mm diameter were punctured in the culture medium. Required concentration of serially diluted extracts (0.6, 1.2, 2.5, 10, 20 and 40mg/ml) were added to the wells. The plates thus prepared were left for diffusion of extracts into media for 1hr in the refrigerator and then incubation for 48hr, the plates were observed for zones of inhibitory. The diameter zone of inhibitory was measured and expressed in millimeters. Dimethyl Formamide (DMSO) was used as a negative control. Ketoconazole used as positive control (500µg/ml). the experiments were conducted in triplicates. The same method was followed for testing antibacterial activity using nutrient agar medium incubated at 37^0C for 18hr.

DETERMINATION OF THE MIC BY BROTH DILUTION ASSAY (NCCLS 1997)

The minimum inhibitory concentration of the plant extract was determined using broth dilution assay. The medium containing different concentrations of plant extracts viz*;* 100mg -1µg per ml prepared by serial dilution (10^{-1} dilution). After inoculation of culture, the tubes were incubated for 72hrs at 28^0C. The MIC of each sample was determined by measuring the optical density in the spectrophotometer (Electronics India) at 520nm and compared the result with those of the non-inoculated broth used as blank. Control was prepared using media and inoculum without plant extract. The

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experiment was conducted according to NCCLS Standards (now called as CLSI) (Ogu-GI-2011 and Shinki S A 2011).

III. RESULTS AND DISCUSSION

In the present investigation five fungal species and six bacterial species were tested to determine the antifungal and antibacterial activity of 98% of methanol leaf stalk extract of P. betle L. the values given in tables-1.3and 1.4,(plate 1.1) are the mean of three observations. The 98% methanol extract showed maximum of (22.00±0.00mm) inhibition in *Candida albicans* at 40mg/ml followed by *Trichophyton rubrum (*14.66±1.15), *Microsporum gypseum* (12.33±1.52mm), *Aspergillus flavus* (11.66 ±1.15mm), and *Trichophyton tonsurans* (10.00±0.00mm). The minimum inhibitory concentrationsof test fungi were determined and the values are givenin figure 1.2. The MIC of *T.rubrum* and *C. albicans* are 0.62 mg/ml conc. Followed by *M. gypseum* 1.25 mg/ml conc., and *A. flavus, T. tonsurans* 2.5 mg/ml conc.

The 98% methanol leaf stalk extract at 40mg/ml conc. showed maximum of (21.00+1.00mm) inhibition against *Escherichia coli* and *Serratia marcescens* followed by *Bacillus subtilis (*18.66+1.15mm*),* Psudomonas aeruginosa (16.33+1.52mm) and the least inhibition zone shows by *Staphylococcus aureus, Brevbacillus brevis* with (15.00+1.00mm). The minimum inhibitory concentrations of test bacteria were determined and the values are given in figure 1.2. The MIC of *E.coli, S.marcescens, B. subtilis, S. aureus, B. brevis* were determined as 0.6mg/ml conc. Followed by *P. aeruginosa* was 1.25mg/ml conc.

The negative control used, DMSO could not show inhibition against all the tested fungal and bacterial strains. Ketoconazole used as standard at conc. 5mg/ml shows antifungal activity 24.00mm whereas streptomycin in used standard bacteria shows inhibition zone in 24.00mm.

Fungal strains	Different conc. (mg/ml) of crude and inhibition zone in mm								
	40	20	10	5	2.5	1.25	0.62	Control (DMSO)	Standard (Ketoconazole)
T. rubrum	14.66±1.15	12.66±0.57	09.66±1.00	08.00±1.00	07.66±1.15	06.00±0.00	06.00±1.00	-	20.66±1.15
M. gypseum	12.33±1.52	10.00±1.00	09.00±1.00	08.66±1.15	06.00±1.00	05.33±1.52	-	-	18.66±1.15
C.albicans	22.00±0.00	13.33±1.52	12.66±1.15	11.00±1.00	10.66±0.57	09.66±1.15	06.33±1.52	-	21.00±0.00
T. tonsurans	10.00±0.00	09.00±1.00	08.00± 1.00	07.00± 0.00	05.00±1.00	-	-	-	24.66±0.57
A.flavus	11.66±1.15	09.66±0.57	07.00± 1.00	06.66± 1.15	05.66± 0.57	-	-		25.66±1.15

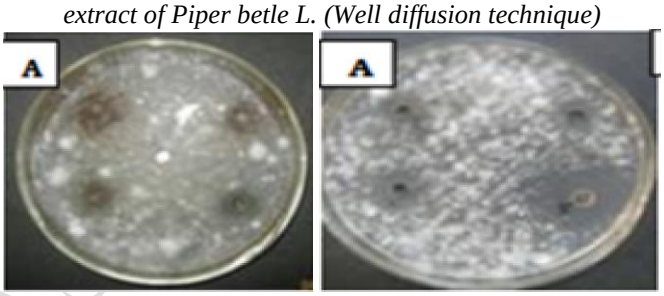
T. rubrum: Trichophyton rubrum, M. gypseum: Microsporum gypseums, T. tonsurans: Trichophyton tonsurans, A.flavus: Aspergillus flavus, C.albicans: Candida albicans, Negative control: DMSO, N,N-Dimethyl Formamide, Standard Ketoconazole (Positive control)

Table 1.3: Antifungal activity of 98% methanolic leaf stalk extract of Piper betle L. (Well diffusion technique)

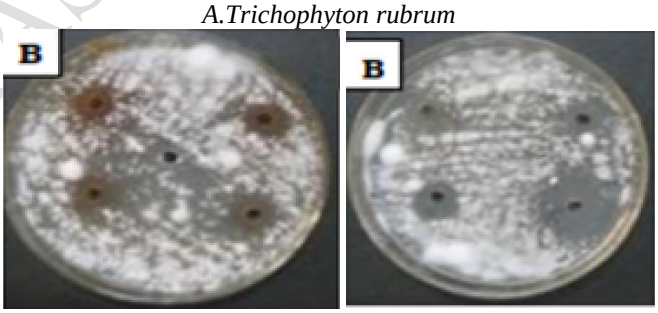
Bacterial Strains	Different conc. (mg/ml) of crude and inhibition zone in mm								
	40	20	10	5	2.5	1.25	0.62	Control (DMSO)	Standard (Ketoconazole)
E. coli	21.00±1.00	20.66±1.15	17.33±1.52	14.00±1.00	12.33±1.52	10.66±1.15	08.66±1.15	-	24.00±1.00
B.subtilis	18.66±1.15	15.33±1.52	14.00±1.00	13.66±1.15	10.33±1.52	09.33±1.52	07.00±1.00	-	20.66±1.15
S.marcescens	21.00±1.00	19.33±1.52	18.00±1.00	16.00±1.00	12.33±1.52	10.33±1.52	0.9.33±1.52	-	24.33±1.52
S. aureus	15.33±1.52	13.00±1.00	10.33±1.52	09.00±1.00	07.00±0.00	06.66±1.15	05.00±1.00	-	18.00±1.00
P.aeruginosa	16.33±1.52	14.33±1.52	13.66±1.15	11.66±1.15	10.33±1.52	08.33±1.52	-	-	16.66±1.15
B.brevis	15.0±1.00	14.33±1.52	13.00±00.0	11.00±0.00	09.00±1.00	08.00±1.00	06.00± 0.00	-	24.33±1.52

E. coli: Escherichia coli, B.subtilis: Bacillus subtilis, S.marcescens: Serratia marcescens, S.aureus: Staphylococcus aureus, P.aeruginosa: Psudomonas aeruginosa, B.brevis: Brevbacillus brevis Negative control: DMSO, N,N-Dimethyl Formamide, Standard Ketoconazole (Positive control).

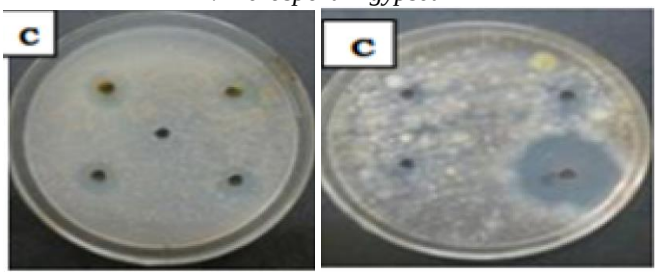
Table 1.4: Antibacterial activity of 98% methanolic leaf stalk extract of Piper betle L. (Well diffusion technique)



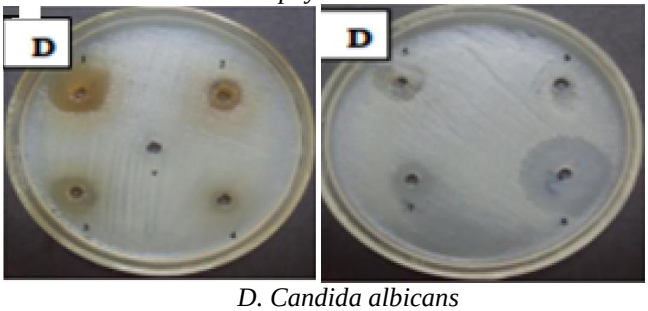
A.Trichophyton rubrum



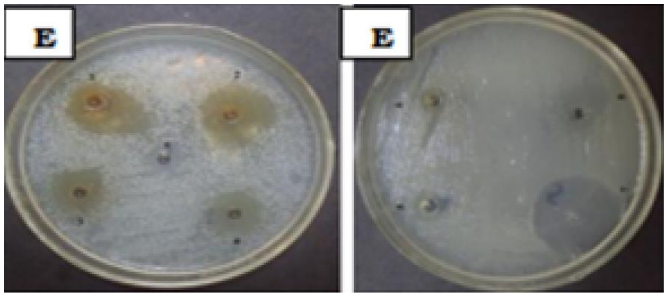
B.Microsporum gypseum



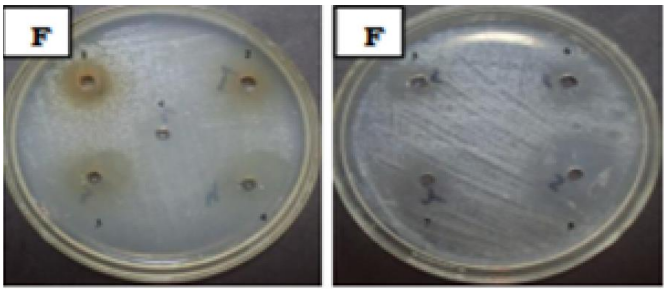
C.Trichophyton tonsurans



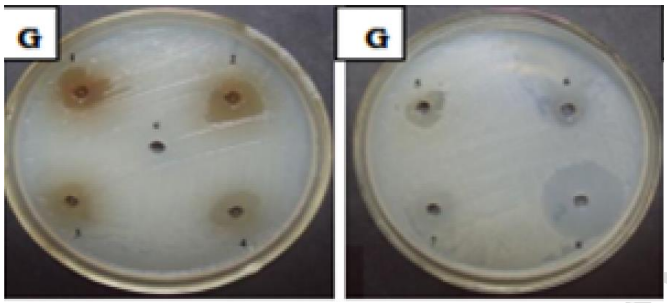
D. Candida albicans



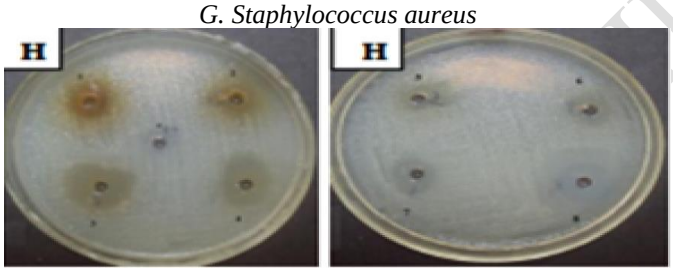
E. E. Coli



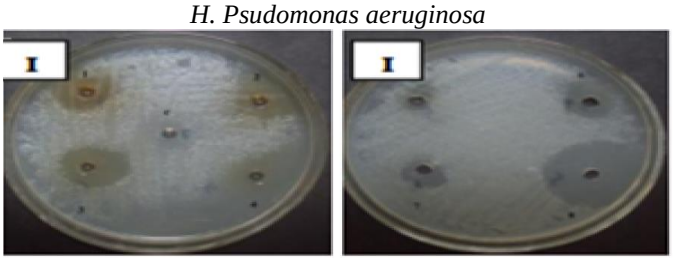
F. B. Subtilis



G. Staphylococcus aureus



H. Psudomonas aeruginosa



I: Brevibacillus brevis

A: Trichophyton rubrum, B: Microsporum gypseum, C: Trichophyton tonsurans, D. Candida albicans, E: E.Coli, F: B. Subtilis, G: Staphylococcus aureus, H: Psudomonas aeruginosa, I: Brevibacillus brevis, 1=40mg/ml, 2=20mg/ml,3=10mg/ml, 4=5mg/ml, 6=1.25mg/ml 7=0.62mg/ml, C=Negative control: DMF N, N- Dimethyl Formamide, 8=Standard: ketoconazole(Positive control against Fungi), streptomycin sulphate (Positive control against bacteria)

Plate 1.1: Antidermatophytic activity of 98% methanolic leaf stalk extract of Piper betle L. (Well diffusion technique)

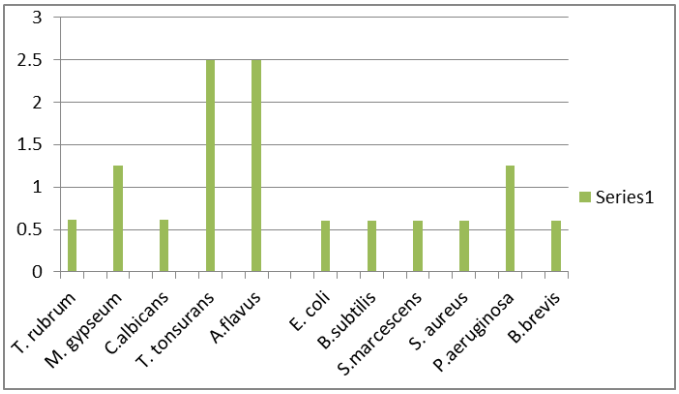


Figure 1.2: Minimum Inhibitory Concentrations of 98% methanolic extract of Piper betle L. against test strains

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