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Research Article

EVALUATION OF FUNGITOXIC POTENCY OF *PIPER BETEL* L. (MYSORE VARIETY) LEAF EXTRACTS AGAINST ELEVEN PHYTO-PATHOGENIC FUNGAL STRAINS

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ABSTRACT
Among plant microbial pathogens like bacteria, fungi, viruses etc., fungi are the most important and prevalent pathogens, infecting a wide range of host plants and are responsible to cause economical losses of crops in field and harvests during storage and transportation. Regulation of fungal pathogens with chemicals, under field condition is not only carcinogenic and hazardous to health but also causes serious environmental pollution due to their non-degradable nature. In addition, their indiscriminate usage has resulted into an induced resistance among the pathogens.
Thus the quest to find the effective, bio-safe and bio-degradable alternative fungicide is the major concern. Different genera plants produce a wide range of Plant Secondary Metabolites (PSMs) or Phyto-chemicals. Apart from routine uses, huge number of plants are not been fully explored for their bioactive properties of secondary metabolites, essential oils and volatile fractions. Thus, PSMs which have defensive role may be exploited for the management of plant diseases. *Piper betel* L is a medicinally important plant and well studied for antimicrobial activity but poorly explored to screen antifungal potency against various plant disease causing fungal strains. In the present study, efforts made to test the antifungal potency of *Piper betel* L leaf extracts prepared in various polarity solvents water, methanol, chloroform and petroleum ether using Paper disc diffusion assay.

Key Words: *Plant Fungal Pathogens, Plant Secondary Metabolites (PSMs), Antifungal Potency, Paper Disc Diffusion Assay*

INTRODUCTION
The plant world is a rich storehouse of natural chemicals. Variety of higher plants contains rich diversity of bioactive PSMs like phenols, flavanoids, quinones, tannins, alkaloids, saponins, sterols and terpenoids, responsible to play a defensive role in the plants. Such plant chemicals contribute to diverse biological activities such as antimicrobial, allelopathic, antioxidant and bio-regulatory properties and these natural products thus can certainly substitute harmful synthetic fungicides for plant disease control (Patel and Jasrai, 2009; Huang *et al.*, 2010; Patel and Jasrai, 2012).
Hence plants used in traditional medicines ought to be scientifically investigated as a potential source of novel antimicrobial compounds.
The fungal inhibition can be due to the limitation of the fungal growth by interfering with the fungal protein production, DNA replication, interference with cellular metabolism, damage to the membrane, following death of the fungal cells.
Antifungal activity of secondary metabolites depends on the method and solvent used for extraction, its concentration and composition (Tripathi *et al.*, 2008). As demonstrated by Kishore *et al.*, (2007) Paper disc diffusion assay provides qualitative information on the efficacy of test compounds, and the method can be used routinely to evaluate antifungal activity of extracts. In the context, present study was sought to investigate the comparative effects of different solvent extracts of *Piper betel* L. leaves (Figure 1) on fungal pathogens and further dose optimization study of the effective fractions using Paper disc diffusion assay.

Figure 1: *Piper betel* L. leaves

Fungal Phyto-Pathogens

Fungi predominantly reproduce by the production of asexual spores, which is a major source of fungal infestation and rapid proliferation. *Aspergillus, Mucor, Rhizopus, Fusarium* spp. known as storage fungi of important cereals, and are also reported to produce harmful mycotoxins/aflatoxins. Aflatoxins are biologically active secondary metabolites and are extremely potent carcinogenic, teratogenic, hepatotoxic, immunosuppressive, allergic in nature and inhibits several metabolic systems (Baiyewu *et al.*, 2007; Lokman, 2010). Plant-based fungal pathogens are responsible to cause severe economic losses to crops and harvested products and make them unsafe for consumtion. A study by Fatima *et al.*, (2009) reported, by and large post-harvest deterioration of fresh fruits, vegetables and other plant products occur during harvesting till the consumption due to infection of various fungi viz., *Alternaria alternata* (causes infection in apple, bell pepper, bitter gourd, bottle gourd, papaya, pear, round gourd, sponge gourd, tomato), *Fusarium solani* (infects melon, papaya, egg plant, cucumber, sponge gourd, tomato) *Aspergilllus flavus* and *Aspergillus niger* (infects lemon, mango, round gourd, tomato). A study by Aye *et al.*, (2009) shows that sheath and stem disease of Rice is caused by *Rhizoctonia* and *Sclerotium* species and harms the Rice production.

Antifungal property of many plants has been studied earlier by many researchers in order to control plant diseases in a bio-safe way. However for *Piper betel* L., most of the studies are carried out to find the antibacterial proterty of the plant. As in, Betel oil tested against yeast and food spoilage bacteria (Panuwat *et al.*, 2006), *Staphylococcus aureus*, *Streptococcus pyogenes*, *Candida albicans* and *Trichophyton mentagrophytes* (Caburian and Osi, 2010), *Piper betel* crude aqueous extract against pathogenic clinical isolates of bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Subashkumar *et al.*, 2013), clinical bacterial isolates typhoid and paratyphoid typhi and salmonella para typhi A and B (Pasha *et al.*, 2013), oral Candida species like, *Candida albicans, C. tropicalis, C. glabrata, C. dubliniensis, C. lusitaniae, C. krusei* and *C. parapsilosis* (Himratul-Aznita *et al.*, 2011), ethanol extract against foodborne pathogens *Escherichia coli* ATCC 25922, *Vibrio cholera* ATCC 6395, and *Staphylococcus aureus* ATCC 25923 (Hoque *et al.*, 2011), ethyl acetate extract against *Staphylococcus aureus, Pseudomonas aeruginosa* (Agarwal *et al.*, 2012). While in one report, *Piper betel* ethanol extract tested against fungi *Alternaria alternata* by Begum *et al.*, (2007). Conversely, the poor antifungal activity screening studies on the plant has inspired the present work and thus an effort was made to find the fungitoxic potency of the *Piper betel* L. extracts.

Medicinal Importance of Plant

Piper betel L is a climber plant and commonly known as Betel vine. The leaves contain essential oil and the plant as a whole found to possess important active phyto-chemical constituents like piperine, chavicol, hydroxychavicol, chevibetol, allylpyrocatechol, carvacrol, terpinene, cineole, cadinene, eugenol etc (Patel *et al.*, 2012). The leaves of the plants are traditionally used as a paan -mouth refresher and have a role in oral hygiene due to presence of anti-microbial components (Bissa *et al.*, 2007). Leaf juice is useful as an eyedrops in painful ophthalmic affections and in nightblindness (Patel *et al.*, 2012). Various studies by

Guha (2006), Rathee *et al.*, (2006), Rowa and Hob (2009) supports the pharmacological and therapeutic properties of *Piper betel*, as a breath freshner, cardiac tonic, antimicrobial, antifungal, antioxidant, carminative, digestive, sialagogue, anodyne, aphrodisiac, CNS depressant, antipyretic, anticarcinogenic, antinitrosation, anti-inflammatory, radioprotective, immunomodulatory, antiplatelet and antithrombotic. Thus Betel leaves are useful for the treatment of boils, abscesses, wound, itches, abrasion, cuts and injuries, ringworm, mastitis, mastoiditis, leucorrhoea, otorrhoea, conjunctivitis, headache, hysteria, cold and cough, dyspnoea, disease of throat, colic, dysentery and constipation, piles, swelling of gum, rheumatism and joint pain.

MATERIALS AND METHODS

The present investigation is to screen antifungal potency of *Piper betel* L. leaves extracts against eleven important plant pathogenic fungi (Table 1). The Mysore, India variety of *Piper betel* L. plant material was purchased from the local market of Vadodara, Gujarat. Plant material washed and air dried under shade (one week). The dried plant parts were finely powdered using electric grinder, sieved (mesh size 500 µ) and extracted in various solvents with polar (water, methanol) to non-polar characteristics (chloroform, petroleum ether). For preparation of extracts in organic solvents, viz methanol, chloroform and petroleum ether, the finely powdered plant material (100 g) soaked overnight in solvent (400 ml) in air tight erlenmeyer flask. The residues were repeatedly extracted (three times) in 200 ml of solvent (Khan and Nasreen, 2010; Patel and Jasrai, 2010). The flask content was filtered through a whatman filter paper (no 1). The filtrate was evaporated to dryness to yield a thick and dark residue. While, for aqueous extract preparation, powdered plant material (50 g) was extracted in 1000 ml of distilled water at 50°C temperature until the volume reduces to half. The content then filtered through whatman filter paper (no 1). The filtrate was evaporated till complete dryness in oven (40°C) (Harborne, 1984; Patel and Jasrai, 2010). Each sample was then transferred to glass vials (6 ×2 cm) and % yield of extracts was calculated. The extracts utilized for screening antifungal activity against eleven phyto-pathogenic fungi. Fungi *Fusarium oxysporum* (MTCC No. 284), *Rhizopus oryzae* (MTCC No. 3690), *Sarocladium oryzae* (MTCC No. 2046) and *Sclerotium hydroophillum* (MTCC No. 2157) were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. While some fungi were isolated from the infected plant material (collected from local markets of Gujarat region) on PDA (Potato Dextrose Agar) media following standardized protocols (Dube, 1990) for the study. Fungi namely, *Alternaria alternata* (GUB01) isolated from apple fruit, *Aspergillus flavus* (GUB02) from peanuts, *Aspergillus Niger* (GUB03) from lemon, *Fusarium oxysporum* f.sp. *laginariae* (GUB04) from bottle gourd, *Fusarium solani* (GUB05) from potato tuber, *Fusarium solani* (GUB06) from tomato fruit and *Rhizoctonia solani* (GUB07) from potato tuber. Fungal cultures were further grown and maintained on SDA (Sabouraud Dextrose Agar) media at 28 ± 2^{o}C.

Determination of in vitro Antifungal Properties of Extracts

Eleven fungal isolates (Table 1) were used for present growth inhibition assay. *Piper betel* different solvent extracts screened for presence of antifungal activity at a selected concentration range using Paper disc diffusion assay (Erturk, 2006) on SDA media. For the bioassay, a fungal broth culture was established on SDA broth medium (25 ml broth/150 ml flask). The spore count of the culture after specific incubation period was performed using Haemocytometer (Table 1). Before the bioassay, the fungal broth culture was macerated and homogenized under sterile condition. This fungal culture (0.1 ml aliquot) with known spore count was uniformly seeded with sterilized cotton swab on SDA media (15 ml, ≈ 4 cm thickness) in each petri dish (90 × 90 mm). Then extract loaded whatman paper discs (6 mm diameter) were placed on the fungal seeded plates with sterile forecep under aseptic conditions. The plates were incubated in upside down position for 72 hr at 28 ± 2°C (Parekh and Chanda, 2007). The experiment was performed in triplicates with appropriate untreated controls. The ZI (zone of inhibition) including disc diameter, measured by the antibiotic zone reader (Labfine, India) in mm (milimeter) unit. The Primary screening was performed using 10 mg/disc concentration and Secondary screening performed at

0.5, 1, 2.5, 5, 8 and 10 mg/disc concentration of extracts to find the MIC value (Minimum Inhibitory Concentration) for each fungi (Huang *et al.*, 2010).

Table 1: Test-fungi, Incubation period and Haemocytometer spore count

Fungi	Stock code	Incubation period (Days) in broth medium*	Fungal Spore count (× 10^6)
Alternaria alternata	GUB01	6	0.72
Aspergillus flavus	GUB02	5	35.64
Aspergillus niger	GUB03	5	16.69
Fusarium oxysporum	MTCC 284	3	7.86
Fusarium oxysporum f.sp. *laginariae*	GUB04	3	4.62
Fusarium solani	GUB05	3	1.33
Fusarium solani	GUB06	3	1.31
Rhizopus oryzae	MTCC 3690	2	5.78
Rhizoctonia solani	GUB07	5	No sporulation
Sarocladium oryzae	MTCC 2046	5	2.39
Sclerotium hydroophillum	MTCC 2157	10	8.40

*[Note: *Sabouraud Dextrose broth- composed of Dextrose- 20g and Peptone 10g/l with pH 6.5]*

RESULTS AND DISCUSSION

The results for presense (+) and absecnce (**-**) of antifungal activity was obtained with Primary screening experiment (Table 2). Extracts with positive effect were subjected for Secondary screening and dose optimization study in the selected concentration range (0.5 to 10 mg/disc). Thereby obtained fungitoxic spectrum was recorded (Table 3), also reffered as MIC value of the extract against test fungi. The primary screening for antifungal activity using Paper disc diffusion assay revealed excellent results. In the study, fungi *Sarocladium oryzae* found most susceptible, and *Rhizopus oryzae* found as most resistant fungal strain. Furthermore, present study is the first report on the control of *Sarocladium oryzae, Sclerotium hydroophillum* and *Rhizopus oryzae* using plant extracts. In fact, very little work has been conducted on the botanical controls for these crop and yeild destructive fungal strains.

Table 2: Primary screening study for Antifungal potential of extracts (10 mg/disc)

Fungi	***Piper betel* Extracts**			
	WE	**ME**	**CH**	**PE**
Alternaria alternata	-	+	+	-
Aspergillus flavus	-	+	+	-
Aspergillus niger	-	+	+	-
Fusarium oxysporum	-	+	+	-
Fusarium oxysporum f.sp. *laginariae*	-	+	+	-
Fusarium solani (GUB05)	-	+	+	-
Fusarium solani (GUB06)	-	+	+	-
Rhizopus oryzae	-	-	+	-
Rhizoctonia solani	-	+	+	-
Sarocladium oryzae	-	+	+	+
Sclerotium hydroophillum	-	+	+	-

*[**Note:** (+) = indicates antifungal activity, (–) = indicates no antifungal activity; **WE**= Water extract; **ME**= Methanol extract; **CH**= Chloroform extract; **PE**= Petroleum ether extract]*

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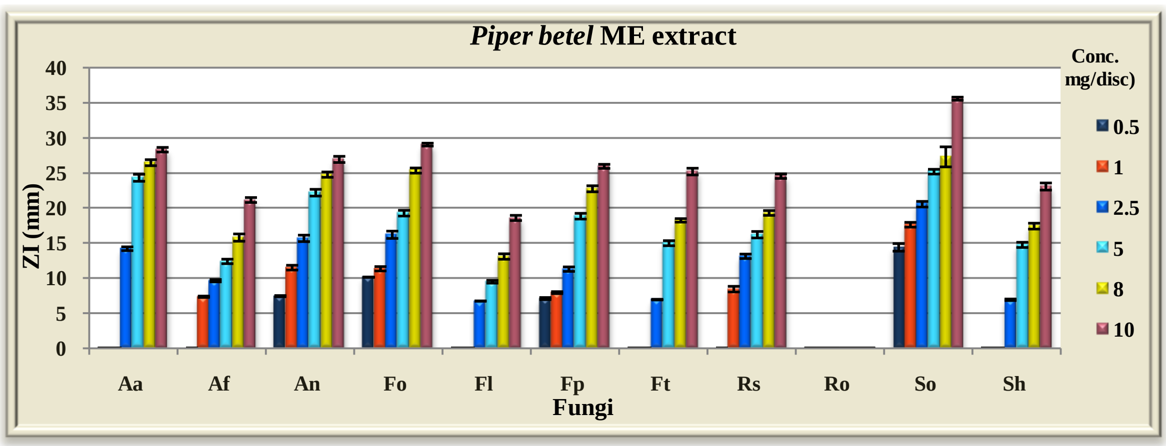
A broad spectrum antifungal activity of *Piper betel* (CH extract), and found to inhibit all the eleven selected fungal strains in the study. While *Piper betel* (ME extract), successfully inhibited ten fungi. While a narrow spectrum inhibitory activity was demonstrated with *Piper betel* (PE extract) which inhibited single fungi *Sarocladium oryzae*, out of the selected fungal strains. Besides *Piper betel* aqueous extract found inffective to inhibit the growth of selected fungal strains at a requisite concentration range.
Pathogen inhibition at lower MIC value /at lesser extract concentration signifies a very effective inhibitory potential. Accordingly the antifungal extracts with well defined MIC value, can be further utilized for the value addition and fungicide development. The secondary screening results demonstrated broad-spectrum and efficient antifungal activity of *Piper betel* (CH and ME extracts) (Table 3).

Table 3: Secondary screening study and overview of obtained MIC value against tested fungi

Piper betel	**MIC (mg/disc)**					
extracts	**0.5**	**1**	**2.5**	**5**	**8**	**10**
ME	An, Fo, Fp, So	Af, Rs	Aa, Fl, Ft, Sh			-
CH	Af, An, Fo, Rs		So, Sh	Aa, Fl, Fp, Ft	Ro	-
PE	-	-	-	So	-	-

*[**Note: Aa** = Alternaria alternata, **Af** = Aspergillus flavus, **An** = A. niger, **Fo** = Fusarium oxysporum, **Fl** = F. oxysporum f.sp. laginariae, **Fp** = F. solani (GUB05), **Ft** = F. solani (GUB06), **Rs** = Rhizoctonia solani, **Ro** = Rhizopus oryzae, **So** = Sarocladium oryzae, **Sh** = Sclerotium hydroophillum]*



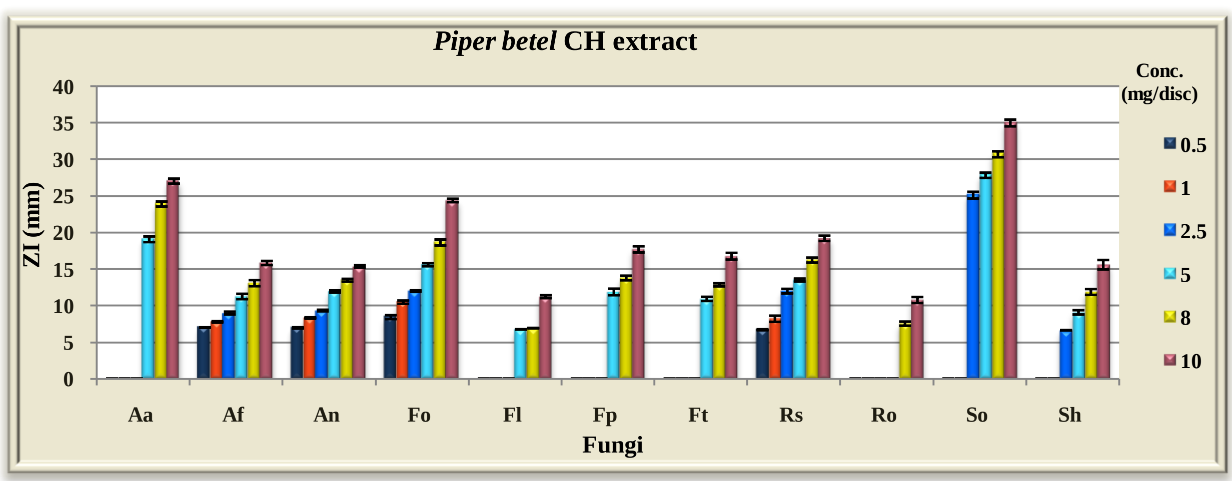


Figure 2: Graphs of extracts demonstating ZI

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In the secondary screening and dose optimization study, *Piper betel* (ME extract) found to inhibit ten fungal strains, indicating its great antifungal potency. At the lowest tested MIC value (0.5 mg/disc), the *Piper betel* (ME extract) found to prevent the growth of *Aspergillus niger, Fusarium oxysporum, Fusarium solani* (GUB05) and *Sarocladium oryzae* with their respective ZI 7.42 ±0.08; 10.12 ±0.31; 7.07 ±0.15 and 14.37 ±0.54 mm. While at 10 mg/disc concentration, the *Piper betel* (ME extract) demonstrated largest and noticeable ZI against fungi *Sarocladium oryzae* (35.58 ±0.22 mm) followed by *Fusarium oxysporum* (29.03 ±0.21 mm) and *Alternaria alternata* (28.3 ±0.33 mm) (Figure 2, 3).

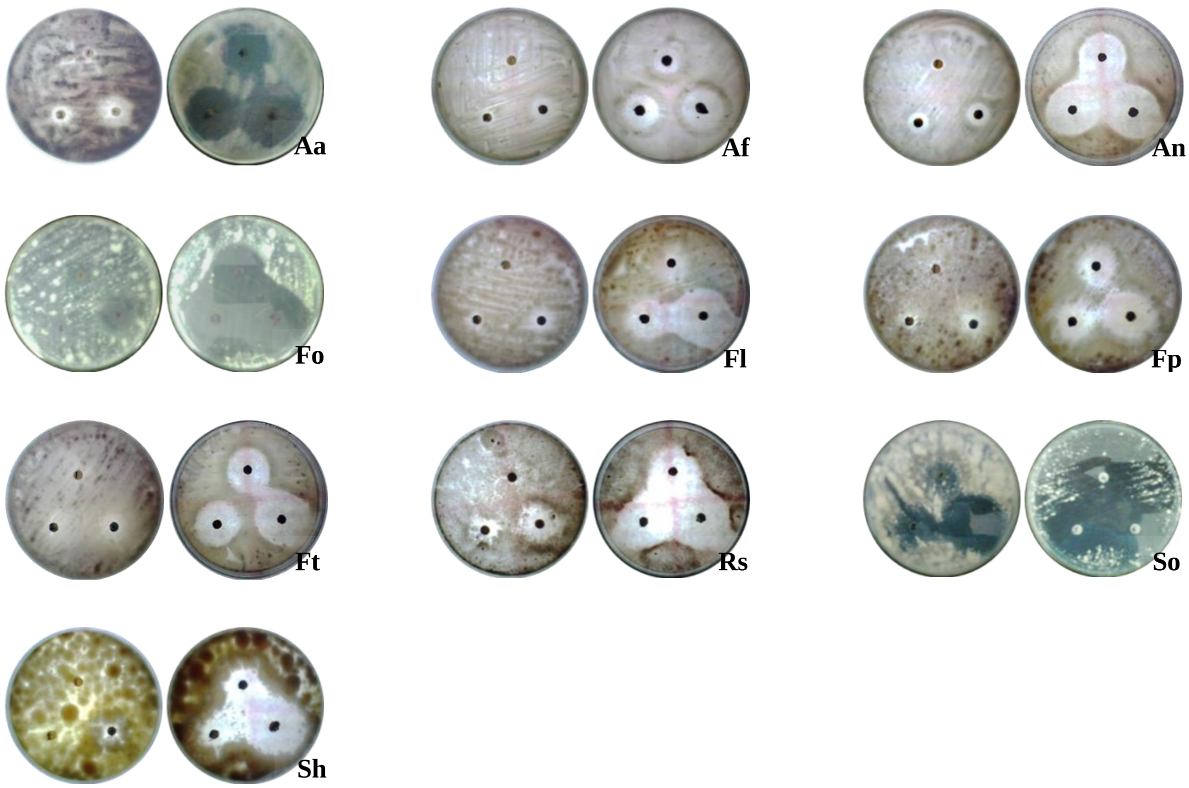

Figure 3: Fungal inhibition by *Piper betel* ME extract

***[Note:** Discs in increasing extract concentration (0.5, 1, 2.5, 5, 8 and 10 mg/disc) from left to right in all figures]*

In case of *Piper betel* (CH extract), at 0.5 mg/disc MIC value, found to restrict the growth of *Aspergillus flavus, Aspergillus niger, Fusarium oxysporum* and *Rhizoctonia solani* with their respective ZI 7 ±0; 6.95 ±0.08; 8.43 ±0.27 and 6.70 ±0.06 mm. While at 2.5 mg/disc MIC the CH extract inhibited fungi *Sarocladium oryzae* and *Sclerotium hydroophillum* with 25.1 ±0.461 and 6.63 ±0.004 mm respective ZI in the study. The CH extract displayed largest and marked ZI against fungi *Sarocladium oryzae* (34.98 ±0.46 mm) followed by *Alternaria alternata* (27.02 ±0.34 mm) at 10 mg/disc concentration (Figure 2, 4).
On the whole, present investigation has noticeably demonstrated the potential antifungal effectiveness of *Piper betel* L. chloroform and methanol extracts. This has proved the broad-spectrum antifungal effect of the selected plant and thus the effective fractions can be further utilized to develop bio-safe herbal formulation.

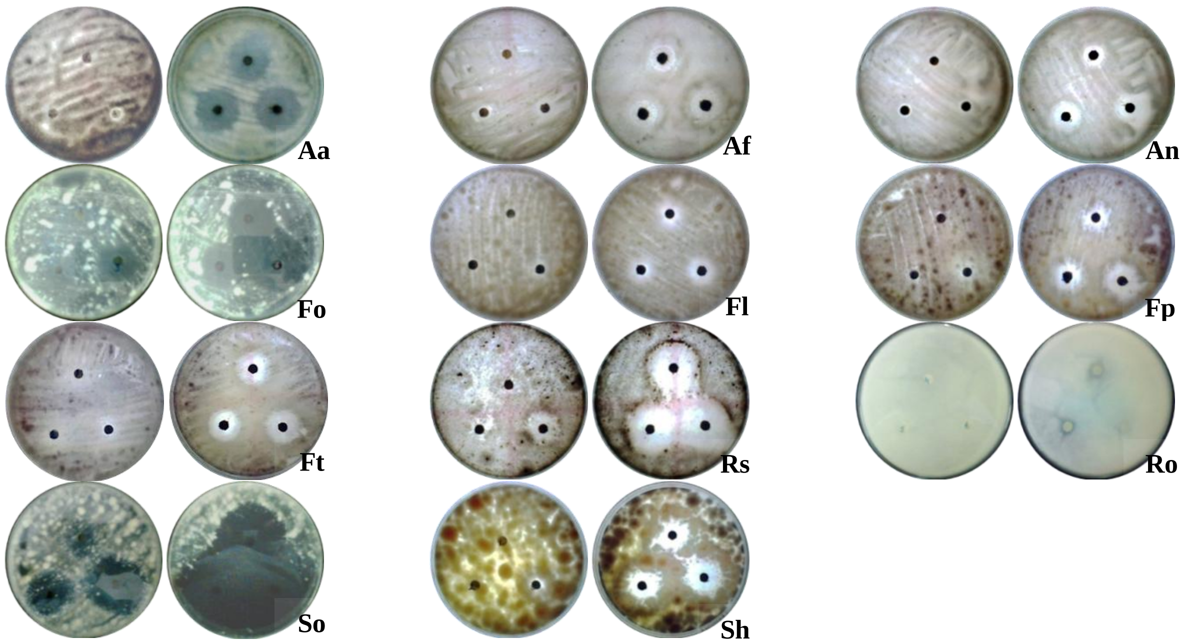

Figure 4: Fungal inhibition by *Piper betel* CH extract

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