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Biochemical profiling of antifungal activity of betel leaf (*Piper betle L.*) extract and its significance in traditional medicine

Sarika Pawar[1#], Vidya Kalyankar[2], Bela Dhamangaonkar[1], Sharada Dagade[3], Shobha Waghmode[2]* and Abhishek Cukkemane[1]

[1]Bijasu Agri Research Laboratory LLP, Sr. No. 37, Kondhwa Industrial Estate, Khadi Machine Chowk, Kondhwa, Pune-411048, India
[2]Department of Chemistry, M. E. S Abasaheb Garware College, Pune - 411 007, Maharashtra, India
[3]Department of Chemistry, Y. M. College, Bharathi Vidyapeeth, Pune-411038, Maharashtra, India
#Present Address: Microbial Diversity Research Centre, Dr. D. Y. Patil Biotechnology and Bioinformatics Institute, Dr. D. Y. Patil Vidyapeeth, Tathawade, Pune-411033, India

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***Corresponding author:** ShobhaWaghmode, Department of Chemistry, M. E. S Abasahaeb garware College, Pune - 411 007, Maharashtra, India; Tel. No: +91-9422-31-6985; E-mail: shobhawaghmode@yahoo.co.in*

Abstract

Piper betle (Linn) commonly called as betel leaf is a widely cultivated plant in the Indian subcontinent. The traditional Indian ayurvedic document describes several of its medicinal properties including as an effective antifungal agent. The present study was conducted to evaluate the secondary metabolite that contributes to its antifungal activity. *Invitro* studies were performed on molds and yeasts on antifungal using fractions obtained from ethyl acetate, hexane and ethanol-methanol extracts by well-diffusion technique. Ethyl acetate extracts showed highest anti-fungal activity. Using biophysical techniques such as Nuclear Magnetic Resonance and Fourier transform infrared spectroscopy techniques; we identified the molecule as derivative of the phenyl propanoid family akin eugenol. The molecule can be readily purified using a 2 step solvent extraction procedure along with silica column chromatography. These findings reveal the antifungal and possible commercial potential of the plant extract and its potential in agriculture against pest management and food spoilage.

Aim: To determine the antifungal activity of *Piper betle* L. extracts.

Keywords: Antifungal; Ayurveda; Fourier transform infra-red spectroscopy; Nuclear magnetic resonance spectroscopy; Food spoilage; Agriculture.

Introduction

In recent years, the increase in resistance in known fungal pathogens to the available antifungal drugs has raised enormous challenges to public health issues [1-3]. In addition, conventional antifungal drugs have undesirable side effects and are very toxic such as chlorhexidine, imidazole and amphotericin B [4]. One important demand of critical significance in this context is to search for novel antifungal agents that would be less toxic and more effective. Interestingly; several medicinal plants have been extensively investigated in order to find novel bioactive compounds [5]. Moreover, several studies have suggested that a number of plant species possesses promising antimicrobial compounds [6-9]. *Piper betle* Linn. (*Piperaceae*), a slender creeping plant, is widely distributed in India, Sri Lanka, Thailand and other tropical countries. This plant has deep green heart shaped, smooth, shinning and long stalked leaves, with pointed apex. Betel leaf possess strong aromatic flavor and have been long in use for the preparation of traditional Indian ayurvedic herbal remedies. It has been reported for the treatment of various diseases such as conjunctivitis, boils and abscesses, cuts and injuries etc. [5,10]. In addition, it also acts as a breath freshener, a digestive and pancreatic lipase stimulant and a pain killer in joint pain [11,13]. Even though all these positive effects of betel leaf are known, the biochemicals of these favorable effects remain obscure. Moreover, betel leaf extract has previously been shown to have strong antimicrobial effects in review [14-19]. A couple of research articles [20,21] have demonstrated the potential of the leaf extract on dermatophytes. Here in, we show that the leaf extract possess antifungal activity against various plant pathogens. The present study was sought to investigate the effects of ethyl acetate, hexane and ethanol-methanol extracts of this plant leave on fungal pathogens.

Here in, we employed biophysical techniques to identify the active metabolite that provide the betel leaf extract with its anti-fungal and other pharmacological properties that have been valued in traditional Indian ayurveda.

Materials and methods

Betel leaf extract

The crude extract and isolation was done using Silica Gel (100-200 mesh) for column chromatography and HPLC grade solvents. Betel leaf was collected from Western Maharashtra farm (Indapur tehsil), India. It was cleaned first with distilled water

and dried in shadow for a week. The dried leaves were powdered and 100 g was used for extraction. The powder was transferred into 1 L conical flask with 500 mL ethanol to completely soak the powder, which was incubated at room temperature for 24 hours. The sample was filtered using ordinary filter paper directly into the clean round bottom flask and set for distillation. We obtained up to 30 mL of distillate from a single run under controlled heating at 55 ° C under reduced pressure. We repeated the procedure 3 times and pulled the collected distillate containing crude extract. The crude extract was concentrated on a water bath at 55 ° C under reduced pressure.

During the extraction optimization step, we added 100 mL ethyl acetate/ethanol-methanol/ hexane to the concentrated 100 mL crude extract and mixed well. The mixture was separate during separating funnel and the ethyl acetate/n-hexane fraction was collected in another conical flask. All 3 extracts were concentrated in procedure mentioned above and was further purified using column chromatography using the respective solvent .The eluent was pooled and analyzed by thin-layered chromatography (TLC), Silica gel 60 F254, preloaded Silica gel on Alumina sheets with ethyl acetate as the solvent and observed under UV chamber and Iodine vapors.

Fungal strains and growth conditions

We tested the effect of betel leaf extract on opportunistic fungal pathogen *Aspergillus niger,* which is the causative agent of black mold in several fruits and vegetables [22]. We also isolated saprophytic and opportunistic pathogenic fungi *Rhizopus sp*. [23] from the leaves of *Murraya koenigii.* (curry leaves). Lastly, we isolated a mold from the leaf of figs infested with rust, which was not obligate parasite *Cerotelium (Physopella) fici* [24] but we have tentatively identified it as wild *Aspergillus* sp. These cultures were cultured and maintained on Potato Dextrose Agar (PDA- HIMEDIA) at 4 ° C. A stock inoculums spore suspension of each fungal culture was prepared from fresh, mature (3-daysold) cultures grown on potato dextrose agar plates at 28 ° C.

In-vitro antifungal assay

Antifungal activity of betel leaf extract was tested against *A.niger,* wild *Aspergillus sp.* and *Rhizopus sp.* of the ethyl acetate, hexane and ethanol-methanol extracts of betel leaf sample was tested by well diffusion method. In brief, 500 µL of fungal spore suspension was added to 20 mL PDA medium and poured in petri dish. After solidification, wells of 5mm in diameter were made on this plate. Each well was filled with 50 µL of ethyl acetate, hexane and ethanol-methanol herbal extract. Potassium tellurite was used as positive control and ethyl acetate, hexane and ethanol-methanol solvent used as negative control. The antifungal assay plates were incubated at 30 ° C for 36h. The antifungal activities of the extracts were determined by measuring the diameter of the inhibition zone in millimetres (mm).

NMR and FTIR

The IR spectra of neat sample were recorded on Nicolette iD5, Thermo scientific at room temperature. Standard[1] H NMR spectra was recorded on Jeol 200MHz using $CDCl_3$solvent and TMS (Euriso-top) as a reference at room temperature.

Results

Isolation of active ingredient from *Piper betle* extract

The beetle leaf extract was purified to homogeneity. During the solvent extraction steps, we observed three spots on the TLC plates. After solvent extraction the crude extract was concentrated and run on a column containing 100-200 mesh Silica Gel and Sodium Sulfate for further purification. In all three solvent types that we tested we observed a single spot from there fraction.

Antifungal susceptibility assay

In the next step, we studied the effect of the organic extract against a range of bacterial and fungal cultures. In all tested cases, we observed antimicrobial effect of the extract against fungal cultures only. This is in stark contrast to preciously reported data where few groups have observed anti-bacterial effect of the *Piper betle* extract [14,17,25,26]. Results obtained in the present study revealed that the ethyl acetate extract possess effective antifungal activity against all the tested fungal cultures (Table-1, Figure-1).The highest antifungal activity of ethyl acetate extract was observed for *A.niger* and Black rust followed by *Rhizopus sp*. Antifungal activity of hexane extract was also significant against *A.niger* (5 mm) and *Aspergillus* sp. (8 mm), while ethanol-methanol extract was ineffective against any of the tested fungal cultures. In addition to the molds, we also tested the effects of wild yeasts that were isolated from leaves. We again noticed a strong anti-fungal activity against them. From all the preparations, ethyl acetate fraction provided the best results. This could be because the extraction process is more efficient and perhaps also stable over other solvent.

Biophysical characterization of the extract

Table 1

	Inhibition zone (mm)			
Fungal culture	**Ethyl acetate**	**Hexane**	**Ethanol-Methanol**	**K-tellurite (+ control)**
Aspergillus niger	28	5	-	6
Aspergillus sp.	28	8	-	7
Rhizopus sp.	23	-	-	-

Antifungal activity of ethyl acetate, hexane and ethanol-methanol herbal extract against indicated fungi.

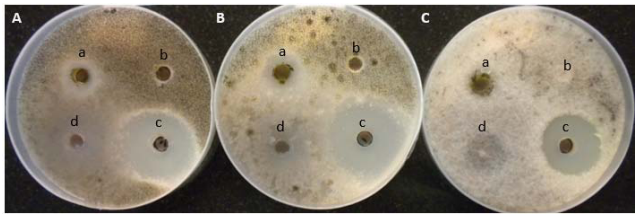

Figure 1: Antifungal activity of ethyl acetate, hexane and ethanol-methanol extracts of herbal sample against A) *A.niger* B) *Aspergillus* sp. and C) *Rhizopus*. Extract preparation in a) Hexane, b) Ethanol-Methanol, c) Ethyl acetate and positive control d) Potassium tellurite

We performed NMR and FTIR spectroscopy in order to understand the functional groups associated with the isolated active compound. In the ^{1}H-NMR spectrum, we observed three types downfield peaks at 6.7, 7.2 and 7.25 ppm for aromatic ring. Additionally plenty of peaks were observed for protons associated with polar and aliphatic groups in the 3.25-5.5 and 1-2.25 ppm, respectively. From the characteristics of the spectra and the results published by other groups, the extracted compound is a derivative of the phenyl propanoid family to which antimicrobials eugenol-chavicol belong [27]. The FTIR spectra well corroborated with the NMR spectrum. We observed a broad peak in the range of 3700 to 3000, which corresponds to absorption caused by N-H, C-H and O-H single bonds. We did not observe any peaks characteristic of triple bonds in the range of 2,500 to 2,000. A wide range of double bond specific groups in C=O and C=C was observed in the region from 2,000 to 1,500. Lastly, the region from 1700-600 show finger print that is reminiscent of phenyl propanoid eugenol [27,28].

Discussion

The antifungal activity of the various extract highlights that ethyl acetate based extraction process provided the best for antifungal property. Surprisingly, unlike other results where *Piperbetle* extract that showed both anti- bacterial and fungal activities, we did not observe any anti-bacterial effect against gram positive *Staphylococcus aureus* and *Bacillus* sp.; few gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Xanthomonas campestris*. This may be due to the fact that our purification method is different to the ones reported before (Table 2). In all previous studies crude extracts have been used hence many groups have reported anti-bacterial, anti-fungal and in some cases anti-cancer effects as well. In our methodology, we have enriched the phenyl propoanoid derivative in the extract by first ethanolic extract and then treating with other organic solvents like ethyl acetate/ hexane/ ethanol-methanol. Furthermore, the large zone of clearance that we observe highlights the better extraction and stability of the phenyl propanoid in the ethyl acetate fraction.

Table 2

Active compound	Extraction method	Result
Hydroxychavicol	choloroform extract	anti-fungal (Ali et al., 2010); antibacterial (Sharma et al 2009)
	hydrodistillation	anti-bacterial (Basak and Guha, 2015)
	Concoction- boiling extraction	anti-cancer (Gundala et al., 2014)
Eugenol	ethanol or water-soxhlet extraction	*Streptococcus mutans* (Deshpande and Kadam, 2013)
	solvent extraction: methanol, ethyl acetate and petroleum ether	*Streptococcus mutans* (Deshpande and Kadam, 2015)
	hydro-distillation	anti-bacterial (Sugumaran et al., 2011)
Hydroxychavicol and Eugenol	methanolic extract	anti-cancer (Paranjpe et al., 2013)
	liquid-liquid and supercritical fluid extraction	Comparison of extraction methods(Singtongratana et al., 2013)

Summary of bio-active extraction procedures from *Piper betle*

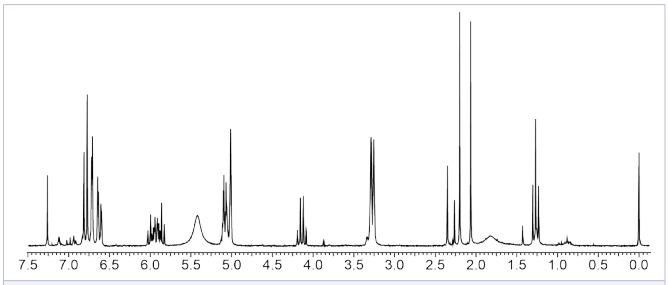

Figure 2: 1D ^{1}H-NMR spectrum of the active ingredient of *Piper betle* extract is suggestive of molecule from the phenyl propanoid- eugenol family

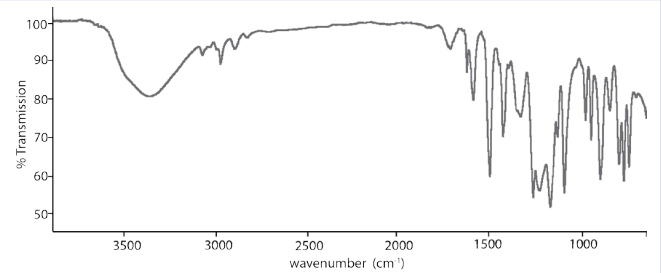

Figure 3: FTIR spectrum of the active ingredient of *Piper betle* extract.

Conclusion

Our finding sheds light on one of the important biochemical metabolite that contributes to the significance of betel leaf in traditional Indian ayurvedic medicine. The metabolite can be readily extracted in a two-step process using organic solvents and silica column chromatography. The active ingredient belongs to the phenyl propanoid family belonging to eugenol-chavicol group have been shown to possess strong antimicrobial properties [15,26,29,30]. More importantly, our results highlight the potential of using betel leaf extract as a potent anti-fungal agent for farming and perhaps also food storage against different types of molds. Food spoilage is a major agricultural problem accounting for heavy losses; therefore it is necessary to make the process commercially viable and efficacious against various pathogens and food spoilage organisms [31,32].

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