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In Vitro Antimycotic Activity of Four Medicinal Plants Versus Clotrimazole in the Treatment of Otomycosis: A Preliminary Study

ABSTRACT

Objective: To determine the antimycotic activity of the four medicinal plant extracts, ***kalachuchi*** bark (*Plumeria acuminata Ait.*), ***atsuete*** bark (*Bixa orellana Linn.*), ***akapulko*** leaves (*Cassia alata Linn.*), and **neem** leaves (*Azadirachta indica Adr. Juss*), when compared to the standard clotrimazole in the treatment of otomycosis.

Study Design: Experimental Study

Methods: Taxonomically identified plants, ***kalachuchi, atsuete, akapulko***, and **neem** tree were collected and deposited in an herbarium. Extracts of these plants and the standard clotrimazole were tested against isolates of *Aspergillus flavus, Aspergillus niger, and Candida albicans* taken from patients with otomycosis. Three trials were made for each extract using different solvents and results subjected to statistical analysis.

Result: Of the four medicinal plant extracts studied, only ***kalachuchi*** bark extract exhibited antifungal activity against *Aspergillus flavus and Aspergillus niger* using methylethylketone as solvent when compared to the standard clotrimazole. It was equally effective in inhibiting the growth of *A. flavus* and *A. niger*. However, all plant extracts using all types of solvents were equally ineffective in inhibiting the growth of *Candida albicans.*

Conclusion: This in vitro study suggested that ***kalachuchi*** (*Plumeria acuminata Linn.*) bark extract inhibits the growth of *Aspergillus* species and was comparable to the standard clotrimazole. Following appropriate further studies and clinical trials, it may be a potential alternative treatment option for otomycosis caused by *Aspergillus* species.

Key words: otomycosis; ***kalachuchi*** (*Plumeria acuminata Linn.*) bark; antimycotic; *Aspergillus flavus*; *Aspergillus niger*

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Otomycosis, a fungal infection of the external auditory canal, is found throughout the world. Its prevalence is greatest in hot, humid, and dusty areas of the tropics and subtropics. Although a wide spectrum of fungi are involved, *Aspergillus* is the most common.[1] Studies by Geaney[2] and by Lakshmipathi and Murti[3] attributed all observed cases to either *Aspergillus or Candida* species.

Several studies have cited the antifungal properties of certain medicinal plants. Of the 10 medicinal plants recommended by the Department of Health (DOH) of the Republic of the Philippines, ***akapulko*** (*Cassia alata Linn*.) showed antifungal activity[4]. A study of 10 medicinal plants in the priority list of the Philippine Council for Health Research Development (PCHRD) and Plant Resource of South East Asia (PROSEA) by Penecilla *et al*[5] concluded that ***kalachuchi*** (*Plumeria acuminata Ait*.) and ***atsuete*** (*Bixa orellana Linn*.) had the highest activity in the assays against *Candida* species. Biswas *et al* observed that the extracts of **neem** leaf and **neem** seed oil kernels are effective against certain fungi including *Trichophyton, Epidermophyton, Microspor, Trichosporon, Geotricum and Candida.*[6]

Interested in finding out which among these medicinal plants—***kalachuchi*** (*Plumeria acuminata Ait*.) bark, ***atsuete*** (*Bixa orellana Linn*.) bark, ***akapulko*** (*Cassia alata Linn*.) leaves, and **neem** (*Azadirachta indica Linn*.) leaves, had fungicidal properties against the common fungal pathogens causing otomycosis, our study aimed to determine the antimycotic activity of these four medicinal plant extracts when compared to the standard Clotrimazole in the treatment of otomycosis.

METHODOLOGY

A. Collection and Identification of Plants

Collection and taxonomical identification of the following plant species was performed: ***kalachuchi*** (*Plumeria acuminata Ait*.) bark, ***atsuete*** (*Bixa orellana Linn*.) bark, ***akapulko*** (*Cassia alata Linn*.) leaves, and **neem** (*Azadirachta indica Linn*.) leaves. A minimum of 500 grams of each species were collected and air dried for three to five days at the West Visayas State University herbarium.

B. Extraction of Plant Material

About 500 grams of each dried plant material (***kalachuchi, atchuete, akapulko,*** and **neem**) were crushed using mortar and pestle. The solvents hexane, methylethylketone and ethanol were used to serially extract the organic constituents from the plants.[7] A 30-gram sample of each was taken for extraction using hexane, methylethylketone, and ethanol respectively. The sample which was dissolved using hexane was macerated for 48 hours with constant shaking using a mechanical shaking bath BT25 (Yamato, Japan). The mixture was then filtered using No. 33 filter paper (Whatman, U.S.A.) and evaporated to dryness using a Heidolph vv 2000 rotavap (Heidolph, Germany) machine. The solid marc was dissolved in another solvent, methylethylketone, and the same process of shaking, filtering, and evaporation to dryness was done. This process was repeated using ethanol. Extracts were stored in 10 ml amber bottles and labeled properly.

C. Laboratory Testing

1. Gathering of Fungal Strains

With informed consent, specimens were taken from patients diagnosed with otomycosis at the Out-Patient Department of a government tertiary hospital by a single otolaryngology resident using sterile cotton swabs and sterile saboraud's dextrose broth tubes. Sample specimens were incubated in complete darkness at room temperature for four days to one week. Tubes were then examined for presence of surface growth or the appearance of mycelial growth structures and spores.

2. Identification of Fungal Species

Only two species of fungi were isolated and identified – *Aspergillus flavus and Aspergillus niger*. Pure isolates of *Candida albicans* taken from the microbiology laboratory were obtained to represent Candida species in order to measure the efficacy of the plant extracts against Candida causing otomycosis.

3. Preparation of Pure Culture and Agar Disks

Pure cultures were prepared using the agar-blocked method.[8]

4. Bioassay Proper

The agar disc method was performed against pure isolates of *Aspergillus flavus, Aspergillus niger and Candida albicans*. The agar disc size was 14.5 mm in diameter. Three trials were made for the control group and for each extract using different solvents. A 100% concentration of the positive control (clotrimazole) was used. Examination of all plates for any zone of inhibition formation was performed. The diameters of the zones of inhibition were

Table 1. Mean Zones of Inhibition Between the Different Treatment Groups against Fungal Pathogens

FUNGAL PATHOGEN	EXTRACTS	SOLVENT	MEAN ZONES OF INHIBITION (in mm.)	
			INITIAL (n=3)	FINAL (n=3)
Aspergillus flavus	Kalachuchi bark	Hexane	14.5	38.5
		MEK	14.5	**46.17**
		Ethanol	14.5	34.83
	Atsuete bark	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	35.33
	Akapulko leaves	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Neem leaves	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Control (Clotrimazole)		14.5	**44.17**
Aspergillus niger	Kalachuchi bark	Hexane	14.5	24.83
		MEK	14.5	**41.17**
		Ethanol	14.5	33
	Atsuete bark	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Akapulko leaves	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Neem leaves	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Control (Clotrimazole)		14.5	**43**
Candida albicans	Kalachuchi bark	Hexane	14.5	14.5
		MEK	14.5	**17.33**
		Ethanol	14.5	14.5
	Atsuete bark	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Akapulko leaves	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Neem leaves	Hexane	14.5	14.5
		MEK	14.5	14.5
		Ethanol	14.5	14.5
	Control (Clotrimazole)		14.5	**22.33**

then measured by getting the average of the zone measuring lengthwise and clockwise.[8]

D. Statistical Analysis

The mean diameter of zones of inhibition of the different treatment groups were compared using analysis of variance in completely randomized design. Pairs of treatment means were compared using the Duncan's Multiple Range Test.

Absence of a statistically significant difference compared to the positive control (clotrimazole) was considered a significant finding.

RESULTS

The positive control (clotrimazole) had a mean zone of inhibition diameter against *Aspergillus flavus* of 44.17 mm. Among the medicinal plants, ***kalachuchi*** (*Plumeria acuminata Ait.*) using methylethylketone (MEK) as solvent had the highest mean zone of inhibition diameter against *Aspergillus flavus* of 46.17 mm. Other extracts showed no increase in the mean zone of inhibition diameters against *Aspergillus flavus.* (Table 1)

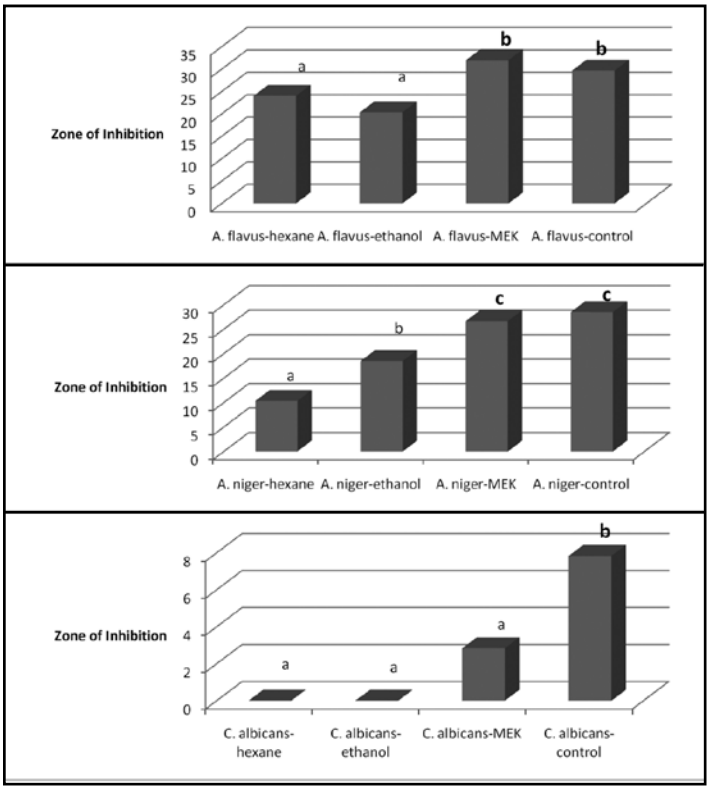
The zones of inhibition using the control, clotrimazole for *Aspergillus niger* were 43.00 mm mean diameter. ***Kalachuchi*** extracts using MEK had mean zone of inhibition diameter at 41.17 mm. Other extracts showed no increase in the mean zone of inhibition diameters against *Aspergillus niger.* (Table 1)

Clotrimazole had only a mean diameter zone of inhibition at 22.33 mm for *Candida albicans*. ***Kalachuchi*** bark extract using only methylethylketone as solvent had only a mean diameter zone of inhibition of 17.3 mm. The three remaining medicinal plant extracts (***atsuete, akapulko,*** and **neem**) using all three solvents exhibited no increase in the mean zone of inhibition diameters against *Candida albicans*. (Table 1)

Since only ***kalachuchi*** extract showed a comparable zone of inhibition diameter to the control (Clotrimazole), it was the only extract that was statistically analyzed.

Statistical analysis using analysis of variance showed that the positive control (clotrimazole) was effective for *Candida albicans* compared to ***kalachuchi*** extract using different solvents. On the other hand, ***kalachuchi*** extract using methylethylketone (MEK) as solvent exhibited the same response as clotrimazole against

Figure 1. Response of Control (Clotrimazole) and *Kalachuchi* Extract using Different solvents against Fungal Pathogens



*Solvent with the same letter showed no significant difference.

Aspergillus flavus and *Aspergillus niger*. (Figure 1)

DISCUSSION

Of the four plant extracts studied, only ***kalachuchi*** bark extract exhibited antifungal activity against *Aspergillus flavus and Aspergillus niger* using methylethylketone as solvent when compared to the standard clotrimazole.

Kalachuchi (*Plumeria acuminata Ait*.) belongs to the family *Apocynaceae*. *Plumeria* species have formally been investigated for isolation of a variety of iridoids and triterpenoids, which exhibited algicidal, antibacterial, cytotoxic and plant growth inhibitor activity. A study by Pandey *et al* revealed that repeated column chromatography of the methanolic extract of the bark of *Plumeria* species obtained the purified iridoid-saccharide plumieride compound.[9] Plumieride may be responsible for the antimycotic activity of ***kalachuchi*** in this study.

This *in vitro* study suggested that ***kalachuchi*** bark extract inhibits the growth of *Aspergillus* species and was comparable to clotrimazole. Following appropriate further studies and clinical trials, it may be a potential alternative treatment option for otomycosis caused by *Aspergillus* species. However, it cannot be overemphasized that the key to successful treatment of otomycosis is gentle efficient cleaning of the ear canal, carefully removing its accumulated debris, thorough drying and application of antifungal agents.[10]

Further research can validate the results of this *in vitro* study by ascertaining specific chemical properties through fractionation and structural elucidation of ***kalachuchi*** bark extract. Toxicity studies and *in vivo* trials can then determine ***kalachuchi*** bark extract's benefit as a topical antifungal agent against otomycosis.

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