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Investigation of the antifungal activity of some non-antifungal drugs in clinical isolates of otomycosis: In vitro study

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Summary:

Otomycosis is one of the commonly encountered problems in otolaryngology practice. Treatment of otomycosis is challenging, and requires a close follow-up; in addition treatment with classical topical or systemic antifungal drugs might be associated with problems of resistance, mucosal irritation, and systemic toxicities, such problems necessitate finding new approaches and cellular targets that can be attacked by new drugs. Proton pump, calcium channels and mitotic spindle are potential targets. Aim of this study is to investigate the antifungal activity of some non-antifungal drugs, namely lansoprazple (proton pump inhibitor), verapamil (calcium channel blocker), albendazole and mebendazole (antihelminthic drugs) against clinical isolates of otomycosis.Ear swaps were collected from 18 patients with otomycosis attending otolaryngology outpatient clinic-Al-Habboobi Hospital. Fungal identification was done depending on microscopical and colonial morphology. Antifungal effect of the tested drugs was assessed by agar well-diffusion assay. Eighteen samples were examined, 13 (72%) of them were positive for fungal growth, 6 were Aspergillus niger , 1 was Aspergillus flavus, 2 were Candida albicans, and the other 4 were Non- albicans Candida.lansoprazole, verapamil, albendazole and mebendazole showed significant antifungal activity at the tested concentrations against Aspergillus niger, Aspergillus flavus, and fluconazole resistant Candida albicans and Non- albicans Candida.

Introduction:

Fungal infections; in general; have long been a major therapeutic challenge.The therapeutic arsenal has been limited, and the use of drugs has been restricted due to toxicity or unfavorable pharmacokinetic profiles. Furthermore resistance to antifungal drugs has been observed.Otomycosis is an acute, subacute or chronic fungal infection of the pinna, the external auditory meatus and the ear canal.[1] However the disease may occur in the

middle ear in case of perforated tympanic membrane.[2] Infection is caused by some species of the saprophytic fungi, such as moulds and yeasts; especially *Aspergillus niger*.[3,4] Other etiologic agents include: *A. flavus*, *A. fumigatus*, *Allescheria boydii*, *Scopulariopsis*, *Penicillium*, *Rhizopus*, *Absidia* and *Candida* Spp.[4-6] In addition, otomycosis is a secondary infection deals to predisposing factors such as bacterial otitis externa corticosteroids therapy and swimming.[2] The presenting symptoms include: scaling, pain, pruritus and feeling of wet ear. Wax formation is also prominent. Otomycosis can occur in both temperate and tropical environment.[3] the prevalence of disease is greatest in hot, humid and in dusty areas. In addition to ear canal toilet, Antifungal ear drops are of value in treatment of otomycosis[7] .There is no consensus on treatment but evidence supports the use of topical ketoconazole[8] .Clotrimazole and econazole drops are very effective but may be needed for 1 to 3 weeks[9]. Clioquinol is both antibacterial and antifungal and may be used as eardrops with hydrocortisone in the formulation of Locorten Vioform™[10].

The concept of non-antifungal drugs:

There is evidence that some drugs targeted at pathogens other than fungi exhibit antifungal activity. In addition, drugs used for treatment of conditions other than infectious diseases might exhibit antifungal activity since fungi and human cell share common pathways, both being eukaryotic. This is of interest since these drugs might be useful for treatment of fungal infection alone or in combination with classic antifungal drugs.[11]

Potential targets for non-antifungal drugs:

Pumps located in the cell membrane are present in order to control the homeostasis between the intra and extra-cellular compartments. Pumps are also present in the endoplasmic reticulum, as proton pump and calcium pumps. Many of these pumps and channels can be considered as potential targets of new antifungal agents, of these potential targets[11]:

- The fungal proton pump is a highly abundant, essential enzyme in the membrane of *Candida albicans*, *Saccharomyces cerevisae*, *Cryptococcus neoformans and Aspergillus niger*[(12-15)]. It belongs to the family of P-type ATPases, a class of enzymes that includes the Na^+, K^+-ATPase and the gastric H^+,K^+-ATPase. These enzymes are cell surface therapeutic targets for the cardiac glycosides and several anti-ulcer drugs, respectively[16]. Proton pump inhibitors, as omeprazole and lansoprazole, which block these pumps either act as agents with antifungal activity or reverse acquired resistance to azoles[11].
- Calcium and its binding protein calmodulin are known to modulate the proliferation, differentiation and metabolism of a variety of cell type. Calcium pump is involved in regulation of free calcium concentration in the cell. Antiarrhythmic drugs, Calcium channel blockers, antiparasitic drugs, antipsychotics, proton pump inhibitors and immunosuppressive agents all can inhibit the calcium influx through its channels. These channels are present in yeasts and moulds and therefore are potential target for new antifungal compounds[11].
- Microtubules are important components of all eukaryotic cells. They form the mitotic spindle for chromosome segregation. In many cells, they function in motility by forming cilia and flagella, and they also serve as major cytoskeletal elements. Microtubules are formed by the polymerization of cx- and 1-tubulin protein subunits. Several drug groups inhibit microtubule polymerization; however, the benzimidazoles are the most important of these in terms of their clinical applications [17]

The tested drugs:

In this study the following non-antifungal drugs were tested for their antifungal potential in comparison with the commonly prescribed azole antifungal drug; fluconazole.

Lansoprazole is a benzimidazole proton pump inhibitor that inhibits gastric acid secretion by blocking the hydrogen-potassium adenosine triphosphate enzyme system (the proton pump) of the gastric parietal cells. Lansoprazole is widely prescribed for treatment of peptic ulcer, gastroesophageal reflux disease and Zollinger – Ellison syndrome. It may cause gastrointestinal disturbances, dry mouth, blurred vision and B12 deficiency as side effects[18, 19].There are growing evidences that proton pump inhibitors may exert an antifungal action or they may reverse the acquired resistance to the widely used azoles antifungal drugs[11].

Verapamil is a phenylalkylamine calcium channel blocker, it interferes with the inward displacement of calcium ions through the slow channels of active cell membranes; it influences myocardial and vascular smooth muscle cells. It is indicated for treatment of hypertension, supraventricular arrhythmias and ischemic heart diseases, it may cause flushing, head ache, ankle edema and allergic reactions as side effects[18].

Albendazole and mebendazole are benzimidazoles that have been used widely in the treatment of helminthic infection as larval cestode infections, such as echinococcosis and cysticercosis. Like other members of this family, they were shown to bind to the beta-tubulin subunit of microtubules, thus stopping mitosis[20].

Patients and methods:

Eighteen patients attending the outpatient otolaryngology clinic/Al-Habobbi hospital /Thi-Qar province with symptoms suggestive of otomycosis were involved in this study. All patients had one or more of the aural symptoms (itching, otalgia, hearing loss).

Methods:

Clinical sampling:

Otomycosis was diagnosed by careful history taking and otoscopic examination. Ear swabs of the affected ears were collected and sent for microbiological assessment.

Microbiological procedures:

Fungal identification:

Fungal isolates were identified on the basis of colonial morphology, lactophenol cotton blue wet-mount microscopy, and slide culture in accordance with standard procedures. Germ-tube testing and morphology on cornmeal agar were also performed to enable the accurate identification of Candida species[21].The presence of fungal elements in stained smears was re-confirmed by fungal culture. Any kind of clinical materials, especially liquid samples (swabs, pus) should be examined as quickly as possible. Swabs did not require processing and were directly used for culture. Swabs were rolled and inoculated over the surface of Sabouraud's Dextrose Agar with chloramphenicol (SC). Cultures were incubated at laboratory ambient (25-27°C) [22].

Drugs and Test controls:

Lansoprazole (lansoprazole 15 mg capsule-Actavis, Barnstaple-UK), verapamil (Dainstole 40 mg tablet-MBC-Syria), albendazole (zentel 100 mg tablet-Smithkline Beecham Laboratories Pharmaceutiques-France) and mebendazole (vermox 100 mg tablet-Janssen-CilAG-Belgium). Fluconazole (Flucomin capsules 150 mg-mission limited-India) was dissolved in distilled water (DW).Negative control included use of distilled water; although no antifungal activity was noted with it. Positive control included use of a common antifungal drug, fluconazole in a separate well of

each plate to compare the fungal susceptibility to the tested agents.

Agar well diffusion assay:

Plates of Sabouraud's Dextrose Agar media were seeded with a suspension of actively growing fungal cells. Wells of 6mm in diameter and about 2 cm apart were punctured in the culture media using sterile cork borers. A fixed volume of fluconazole, lansoprazole, verapamil, albendazole, mebendazole and DW were loaded in the wells using sterilized dropping pipettes and diffusion was allowed at room temperature for 2 hours. After preincubation, the plates were incubated aerobically at 30 C for 24-48 hours. Antifungal activities were determined by measuring diameter of inhibition zone (DIZ) in mm. each experiment was repeated thrice and the average values of antimicrobial activity were calculated[25].

Determination of minimal inhibitory concentrations (MIC):

To measure the MIC values, concentrations of 10, 20, 50 mg/ml of fluconazole, lansoprazole, verapamil, albendazole, mebendazole were prepared in distilled water. The MIC was defined as the lowest concentration able to inhibit any visible yeast growth. All data represent average of three replicated experiments.

Statistical analysis: data are expressed as average, statistical significance between groups was determined by unpaired student's t-test using $p<0.05$ as a criterion for significance.

Results:

18 samples were examined, 13 (72%) of them were positive for fungal growth, 6(46%) of fungal isolates were *Aspergillus niger* , 1 (8%)was *Aspergillus flavus*, 2(15%) were *Candida albicans*, and the other 4(31%) were *Non-albicans Candida*, as shown in table (1) and figure(1).Lansoprazole, a proton pump inhibitor, inhibits growth of *Aspergillus niger, Aspergillus flavus, Candida albicans and Non- albicans Candida* with average diameter of inhibition zones of 10mm, 19mm, 30 mm, 30 mm respectively, while the average diameters noted with verapamil, a calcium channel blocker were 15mm, 15mm, 10mm, 13mm. albendazole inhibits growth of *Aspergillus niger, Aspergillus flavus, Candida albicans and Non- albicans Candida* with average diameter of inhibition zones of 20.5mm, 42.5mm, 27.5 mm, 20 mm respectively. Mebendazole, another antihelminthic drug, inhibits growth of the mentioned fungi with average diameter of inhibition zones of 21mm, 19mm, 17.5 mm, 19 mm respectively respectively. Fluconazole (5mg/ml), as appositive control inhibits growth of *Aspergillus niger, Aspergillus flavus* with average diameters of 14mm,20mm respectively,while causes no inhibition of *Candida albicans and Non- albicans Candida*, as shown in table(2) and figure(2).Minimal inhibitory concentration (MIC) of lansoprazole, verapamil, albendazol and mebendazol against *Aspergillus niger* were 50, 10, 10, 10 mg/ml respectively, MIC of these drugs against *Aspergillus flavus* were 10, 50, 10, 50 mg/ml respectively, while they were 10, 10, 50,10 mg/ml respectively against *Candida albicans*, MIC of the tested drugs against *Non-albicans candida* species were 10, 50, 20, 10 mg/ml respectively. As shown in table (3).

Table (1): Isolates and percentages of fungal species

Organism	Number of isolates	Percentile %
Aspergillus niger	6	46%
Aspergillus flavus	1	8%
Candida albicans	2	15%
Non- albicans Candida species	4	31%

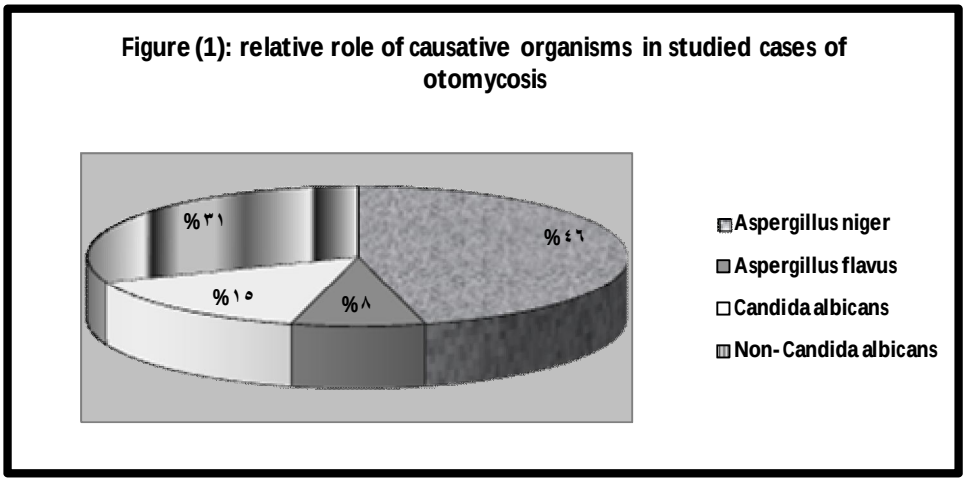

Table (2): Average diameters of inhibition zones (DIZ) for tested drugs in comparison with fluconazole.

ORGANISM	DIZ FOR THE TESTED AGENTS (MM)				
	Fluconazole (5mg/ml)	Lansoprazole (100mg\ml)	Verapamil (100mg\ml)	Albendazole (100mg\ml)	Mebendazole (100mg\ml)
Aspergillus niger	14	10	15	20.5	21
Aspergillus flavus	20	19	15	42.5	19
Candida albicans	0	30	10	27.5	17.5
Non – albicans candida	0	30	13	20	19

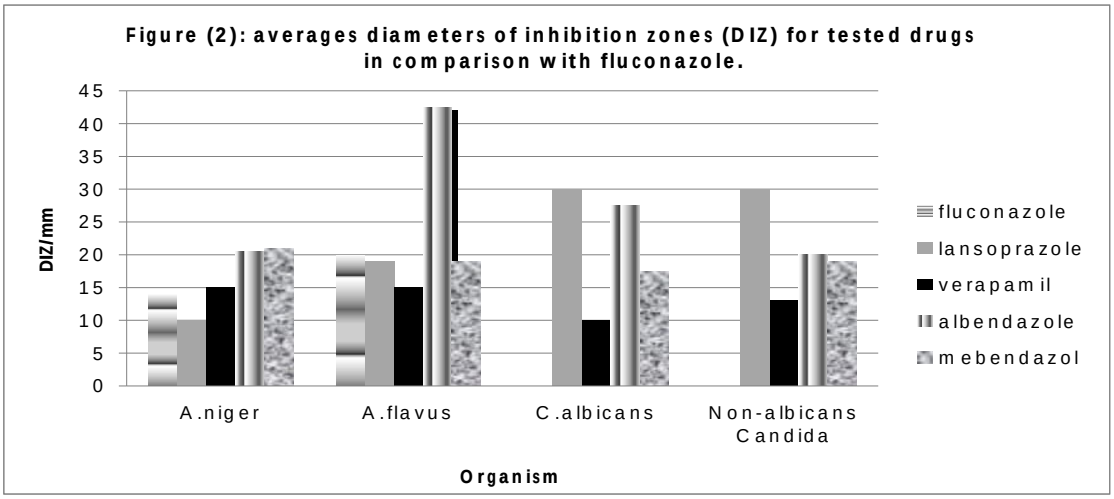

Table (3): Minimal inhibitory concentrations (MIC) of the tested drugs against isolated fungal species.

ORGANISM	MIC FOR THE TESTED DRUGS (MG/ML)			
	lansoprazole	verapamil	albendazole	mebendazole
Aspergillus niger	50	10	10	10
Aspergillus flavus	10	50	10	50
Candida albicans	10	10	50	10
Non – albicans candida	10	50	20	10

Discussion:

Otomycosis is one of the commonly encountered problems in otolaryngology practice. Treatment of otomycosis is frustrating to the patient as well as the otolaryngologist, till now there is no FDA approved antifungal preparation for treatment of fungal otitis externa26. Using antifungal drugs is associated with serious adverse effects as local irritation, hepatotoxicity and nephrotoxicity, in addition to the problem of evolving fungal resistance to the commonly used antifungal drugs as azoles27.Given the increasing incidence of fungal infection and the limited efficacy of currently available antifungal agents, new approaches are needed. A broad spectrum of drugs and natural products might be investigated for their antifungal activity. Review of literatures shows that a considerable number of drugs that are used in clinical practice for indications other than treatment of infectious diseases exhibit antifungal activity by targeting new sites in the fungal cells other than those attacked by azoles and polynes antifungal agents.Lansoprazole, a proton pump inhibitor elicits antifungal effect against the isolated Aspergillus niger, Aspergillus flavus reflected by DIZ which was 10,19mm, Candida albicans and Non-albicans Candida species were susceptible to lansoprazole the DIZ were 30 mm for both. Lansoprazole antifungal activity against the isolated species of Aspergillus was close to that of fluconazole, while lansoprazole activity against candida species is greatly exceeding that of fluconazole which showed no antifungal activity pointing to the problem of antifungal resistance. Such antifungal potential is agreed with some studies in which omeprazole; another proton pump inhibitor; exhibited antifungal activity against saccharomyces28, 29. The exhibited antifungal activity can be explained by lansoprazole ability to inhibit proton pump, calcium pump and to stop mitosis.Albendazole and mebendazole, are benzimidazole that are currently prescribed as antihelminthic drugs, they elicited antifungal activity greater than that of fluconazle at the tested concentrations against the isolated species, as demonstrated by their DIZ against Aspergillus niger (20.5, 21 mm), Aspergillus flavus(42.5, 19 mm), Candida albicans (27.5, 17.5 mm)and Non-albicans Candida(20, 19 mm) . These results agreed with that of N. Berthet 20 who found that Aspergillus spp. Are susceptible to albendazole in vitro and correlate with those obtained on one strain of A. fumigatus by Katiyar & Edlind30. The basis of albendazole action on the pathogenic Cryptococcus neoformans and of other benzimidazole antifungal compounds (benomyl, carbendazim, nocodazole and thiabendazole) on A. nidulans has been well documented31. Mebendazole and albendazole antifungal effect can be attributed to their antimitotic activity.

Verapamil, is a calcium channel blocker, showed antifungal activity comparable to that of fluconazole against Aspergillus niger and Aspergillus flavus species as demonstrated by DIZ which were15mm versus 14mm and 15 mm

versus 20mm. its activity against fluconazole resistant Candida albicans and Non-albicans Candida was evident by DIZ 10,13 mm respectively. Such antifungal potential was evaluated by Krajewska-Kulak E 32 who tested Cinnarizine, verapamil, nifedipine and nimodipine alone or in combination with ketoconazole against clinical isolates of Candida albicans, These drugs alone exhibited antifungal activity in vitro at high concentrations; verapamil was more active than cinnarizine or nimodipine alone. In combination, however, the activity of ketoconazole was potentiated32. Verapamil antifungal activity can be explained by its ability to block calcium channels.

Conclusion:

The tested concentration of lansoprazole, verapamil, albendazole and mebendazole showed significant antifungal activity against the isolated species of Aspergillus and fluconazole resistant Candida. These results supporting the idea of identifying cellular molecules and pumps as new promising targets for new antifungal agents.
Based on these data new approaches to the management of fungal infection in general can be designed.

Recommendations:

Animal studies that take pharmacodynamics into account need to be performed.Clinical assessment of those potential antifungal drugs is recommended. Further investigation of the mentioned antifungal mechanisms in other types of fungal isolates is required. Finding out any synergistic or additive effect of the tested drugs with the classical antifungal drugs both in vitro and in vivo. The role of these drugs or similar drugs in reversing fungal resistance to the commonly prescribed antifungal drugs requires further investigation and analysis.

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تحري الفعالية المضادة للفطريات لبعض الادوية غير المضادة للفطريات في عزلات سريرية لفطريات الاذن:دراسة مختبرية

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الخلاصة:

تعتبر فطريات الاذن من الامراض الشائعة في عيادات الاذن و الانف و الحنجرة . يشكل علاج فطريات الاذن تحديا لكل من الطبيب و المريض لما يستدعيه من متابعة مستمرة بالاضافة الى المشاكل المرتبطة باستخدام الادوية المضادة للفطريات منها مشكلة مقاومة الفطريات للادوية, واحتمال تسبب هذه الادوية بتهييج الغشاء المخاطي للاذن الوسطى, كذلك احتمالية حدوث بعض التأثيرات السامة لهذه الادوية. لذا اصبح من الضروري البحث عن اليات جديدة و جزيئات خلوية يمكن استهدافها بادوية جديدة مضادة للفطريات.هدف هذه الدراسة هو تحري الفعالية المضادة للفطريات لبعض الادوية المستخدمة في علاج حالات اخرى غير الانتانات الفطرية, وهي لانزوبرازول(دواء مثبط لمضخة البروتون),فيراباميل (دواء غالق لقنوات الكالسيوم), البندازول و مبندازول (ادوية مضادة للديدان) مقارنة بالفلوكونازول(مضاد فطري) في عزلات سريرية لفطريات الاذن كخطوة لأيجاد علاج اكثر امان و فعالية من الادوية التقليدية المستخدمة. تم اخذ مسحات الاذن من 18 مريض مصاب باعراض فطريات الاذن يزورون العيادة الخارجية لامراض الانف و الاذن و الحنجرة في مستشفى الحبوبي العام-محافظة ذي قار. تم تشخيص الفطريات المسببة اعتمادا على الشكل المجهري وخصائص المستعمرة. تم تحري التأثير المضاد للفطريات للادوية المستخدمة بطريقة الحفر بالاغار.تم فحص 18 عينة, 13 (72%) كانت موجبة للنمو الفطري, 6 منها اسبرجلس نايغر, 1 اسبرجلس فليفس, 2 كانديدا البيكانس و 4 نون البيكانس كانديدا.وجد ان للادوية المستخدمة(لانزوبرازول, فيراباميل, البندازول و مبندازول) تأثير مضاد للفطريات معتد به في التراكيز المختبرة ضد كل من اسبرجلس نايغر, اسبرجلس فليفس و كانديدا البيكانس ونون البيكانس كانديدا المقاومة للفلوكونازول.