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Research Article

Evaluation of Antifungal Activity of Calvatia Craniiformis and Ivermectin as Novel Alternative Therapies for Aspergillus Niger Associated Acute Otitis Media with Special Refer to SocioDemographic Factors Among Rural Children of Diyala Province-Iraq

Ghassan H Jameel[1], Ali Ibrahim Ali Al-Ezzy[2*]

[1]*Department of Microbiology, College of Veterinary Medicine, University of Diyala, Iraq*
[2]*Department of pathology, College of Veterinary Medicine, University of Diyala, Iraq*

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ABSTRACT

Objectives are to determine antifungal activity of Ivermectin and Calvatia craniiformis as a novel alternative therapy for aspergillus niger associated acute otitis media (AOM) among rural children of Diyala province; correlation of sociodemographic factors with frequency of infection. Ear swabs taken from 58 infected children and cultured on Sabouraud dextrose agar for 7-14 days .Macroscopic and microscopic criteria used for diagnosis of A.niger .High isolation rate for A.niger (27.59%) among children of (4-6) years with significant difference between age groups (p value 0.039); genders (p value 0.004);house status(p value=0.018);family size (p value =0.00006334) and month of infection (p value=0.000). A.niger infection negatively correlated with patients age (p value =0.039), family economy and house status (p value =0.000),family size (p value =0.000). Alcohol extract of *C.craniiformis* (100mg, 200mg, 400mg, 500mg, 600mg, 800mg and 1000 mg) and ivermectin (0.5%,1 % and 2%) restricted the growth of A. niger after 3 days .Significance difference reported between all concentrations except 100 mg and 200 mg ; 600 mg and 800 mg. Significance difference in inhibitory activity between concentration 1% and 2%,0.5% and 2% of Ivermectin respectively. Conclusions: A.niger infections positively correlated with family size and inversely with age and family economy. The growth of *A niger* significantly restricted by alcohol extract of C.craniiformis and Ivermectin in concentration dependent manner. The powerful concentration was 1000mg, for *C.craniiformis* and 2% for Ivermectin. Thus, C.craniiformis and Ivermectin consider a novel antifungal agents that can be used in clinical practice for treatment of *A.niger* associated otitis media that represents a clinical problem in children and need serious attention from clinicians.

Keywords: otitis media; *A.niger*, Ivermectin ;*Calvatia Craniiformis.*

INTRODUCTION

The term otitis media means that there is inflammation of the middle ear. Otitis media can be associated with an infection or be sterile. Bacterial otitis media results from migration of microbes into the middle ear via the Eustachian tube[1,2]. Among bacterial causative for Otitis media *pseudomonas* have the priority in diabetic patients ,in which patients' immune system under stress[3]. Occasionally Otitis media caused by fungi (Aspergillus or Candida) or even viruses such as herpes virus[4-6]. Otitis media is very common in children. It is unusual in adults. The incidence of acute otitis media (AOM) peaks between 6 and 12 months of age, with another lower peak occurring between 4 and 5 years of age[7] . At least one episode of AOM will be experienced by 25-36% of children by age of 1 year[8]. AOM is one of the main reasons for primary care visits, specialist referral, antibiotic consumption and surgical interference procedures among young children[9]. Otitis media is usually diagnosed by the combination of symptoms (ear pain and reduced hearing), and direct observation of an inflamed eardrum with fluid behind it. There is usually fever too[10]. Older children with AOM usually present with a history of rapid onset of ear pain. However, in young preverbal children, otalgia as suggested by tugging, rubbing, holding of the ear, excessive crying, fever, or changes in the child's sleep or behavior pattern as noted by the parent are often relatively nonspecific symptoms[11]. Many authors focused their attention on the bacterial flora or chronic suppurative Otitis media ,but very little is known about the mycological aspect of these. The importance of which has been increasing in the recent years because of broad spectrum antibiotics, corticosteroids, cytotoxic chemotherapy and increase in the number of immune deficiency conditions[12]. *Aspergillus niger* (*A.niger*) is a fungus and one of the most common species of the genus Aspergillus[13]. It is a common contaminant of food[14]. It is ubiquitous in soil and is commonly reported from indoor environments and its black colonies can be confused with those of Stachybotrys[15,16]. Ochratoxins A produced by some strains of *A.niger*[15,17] . Microscopically, *A.niger* conidiophores are smooth-walled, hyaline or turning dark to-

**Author for Correspondence: alizziibrahim@gmail.com*

wards the vesicle. Conidial heads are biseriate with the phialides borne on brown, often septate metulae. Conidia are globose to subglobose (3.5-5.0 um in diameter), dark brown to black and rough-walled[18]. An important focus of AOM prevention is identification of preventable risk factors and subsequent minimization of exposure[19]. To optimize preventive efforts, knowledge on the critical timing of exposures is required[7].

The main objectives of this study are determination of the frequency of *A.niger* as a causative agent for Otitis media in Diyala province; to determine antifungal activity of ivermectin aqueous solution and *C.craniiformis* as a novel alternative therapy for aspergillus niger associated acute Otitis media among rural children; study the correlation of sociodemographic factors such as age, gender, family economy, number of family members with frequency of infection.

MATERIALS AND METHODS

Patients

In this cross sectional, hospital based study, (58) children from rural areas of Diyala province, age range 1-9 years, 40(69%) males,18(31%) females with clinical manifestations of acute otitis media attended to otorhinolaryngology outpatient clinic of Al-Batul teaching hospital for pediatrics and gynecology in Diyala province-Iraq, from January 2014 to December 2014 were studied. Father/Mother were interviewed regarding their socioeconomic condition (Income, housing, total family members)[20].

Current study was conducted according to the principles of Helsinki declaration. A dully filled consent form obtained from all parents of patients participating in the study before taking ear swab. Approval of ethical review Committee of pathology department, College of Veterinary medicine, Diyala University, Iraq was taken prior to initiation of the work.

Methods

Collection of samples

Purulent materials were collected from (58) children suffering from Otitis media. The samples were collected with sterile swabs, which were properly labeled indicating the source; date; time of collection and age of children. After proper collection of samples, swabs transferred to the microbiology laboratory; College of Veterinary medicine, Diyala University within one hour for processing.

Samples processing

Culture

The swab sticks were streaked directly on Sabouraud dextrose agar (SDA) at (25 ± 2) °C and incubated for 7-14 days with continuous observation of growth development[21]. After achievement of fungal growth, morphological features for Aspergillus positive cultures such as colony diameter and color (conidia and reverse) were studied. Microscopic characteristics for the identification were conidial heads color and mycelia[22].

Microscopic Diagnosis

When the mould sporulated, a clean grease free slide was taken for staining. A drop of mounting fluid, lacto phenol cotton blue solution is added on a slide. By sterilized needle, a mycellial mat was transferred on fluid and pressed gently, then mixed with the stain. A clean cover slip had been taken and with the help of a forceps places the cover slip on mycellial mat. Observed under low to high power objectives of microscope[23].

Preparation of Calvatia Craniiformis extract

Body components of *C.craniiformis* mushroom crushed completely after getting rid of dust associated with the mushroom, then brokered a sieve been sifting the powder to get rid of large objects that did not accept the crushing. Five gram of powder placed in a container with 20 ml of methyl alcohol(each 1g dissolve in 4 ml of methyl alcohol) ,then strainer the solution by filter paper in clean and sterile glass tube, then preserve at refrigerator until use.

Antifungal activity of C craniiformis and Ivermectin

The sample is cultured on SDA with the addition of chloramphenicole(0.05ml/dl) and Cycloheximide (Actidione) (0.5mg/ml) to suppress bacterial growth and overgrowth by saprophytic fungi at 35 °C for five to seven days. By using a scalpel, A part of the colony is cut about 1cm of *A.niger*, put on the center of Petri dishes which contain different concentrations of alcohol extract of *C.craniiformis* and ivermectin.

Different concentrations of alcohol extract of *C.craniiformis* were prepared (100mg, 200mg, 400mg, 600mg, 800mg and 1000 mg). Three concentration of ivermectin aqueous solution (0.5%,1 % and 2%) were Prepared. Each treatment consists of four repeat[24-26]

Statistical analysis

Data analysis performed using SPSS for windows TM version 17.0, and Microsoft EXCEL for windows 2010.Frequency of variables express as percentage. Chi-Square and ANOVA used for categorical data analysis, the level of significance was 0.05(two-tail). The level of significant in Pearson and spearman's correlation include also 0.01 (two tail).

RESULTS

Fifty-eight children with Otitis media, mean age (5.10 ± 1.97) years. As shown in table (1), Otitis media was detected clinically among children of(4-6) years (55.2%); (7-9) years(24.14%) and (20.69%) for children (1-3)years. A.niger was isolated from children of (4-6) years (27.59%); followed by (1-3) years (17.24%) and in (10.34%) for children of (7-9) years old. Significant difference was reported regarding the positivity of isolation for the age (1-3) years (p value 0.02). Significant difference was reported regarding the positivity of isolation between age groups (p value=0.039).

Among children presented with otitis media, male represent (69%), and female (31%). A.niger was isolated from (37.93%) of males while (31.03%) was negative. Regarding females, (17.24%) give positive culture for A.niger. Significant difference was recorded in culture positivity between males and females (p value 0.004).

Poor family economy was reported in, 55.2 % compared with (44.8%) represent infected children with good family economy. A.niger isolated from (17.24%) of infected

children with good family economy compared with (37.93%) among those of Poor family economy. No significant difference was recorded regarding family economy and culture positivity. For status of housing for infected children, those resident in old house represent (65.5%) compared with (34.5%) for new houses. A.niger was isolated from (34.48%) of children resident in old houses compared with (20.69%) positive culture for children resident in new houses. Significant difference was recorded in frequency of culture positivity among children resident in new compared with old houses (p value=0.018) .As shown in table (1), the majority of children(58.62%) presented with otitis media came from medium size families followed by those came from large families(27.59%) .The frequency of otitis media was partially low in children from small size families (13.79%). All cases of otitis media for children came from large size families give positive culture for A.niger(27.59%)with significant difference(p value =0.00006334) compared with (20.69%) for those from medium size families. only(6.90%) of otitis media cases give positive culture for those came from small size families.

As shown in table (2), Otitis media was detected more frequently in November(31%) ,followed by September and October (20.7%). Identical frequency (3.4%) for otitis media was reported in January; March; April; May; July and December (3.4%) while in February and August ,otitis media was not recorded. A.niger detected in (55.17%)of otitis media cases. A.niger positive culture was reported in October (17.24%); November(13.79%); September(10.34%) and in June (6.9%).identical frequency for A.niger positive culture was reported in January and December (3.4%).significant difference was recorded regarding culture positivity around months of the year (p value=0.000). No significance correlation between A.niger infection and months of the year(rho = 0.486;p value =0.093).

As shown in table (3), Age has significant negative correlation with A.niger infection (r = -0.272;p value =0.039). Family Economy has inverse correlation with house status (r = -0.513;p value =0.000) and A.niger infections(r = -0.303;p value =0.000). Number of family members has inverse correlation with A.niger infections(r = -0.516;p value =0.000).

Alcohol extracts of *C. craniiformis* concentration (100mg, 200mg, 400mg, 500mg, 600mg, 800mg and 1000 mg) and ivermectin (0.5%,1 % and 2%) restricted the growth of *A niger* at 32_ 35ºC on SDA after2-3 days of incubation (Figure 2, 4). While, no significant difference at 7th day of incubation and the growth of *A. niger* has spread to the surface area of the petri dish as in Figure (3).

Significant difference in A.niger growth inhibition after 3 days of incubation between concentration 200mg and 400 mg; b400 mg and 600 mg; 800 mg and 1000 mg; 100mg and 1000 mg; 200 mg and 1000 mg. Meanwhile no significant difference between concentration of 100 mg and 200 mg; 600mg and 800 mg. No significance difference in inhibitory activity of Ivermectin after 3 days of incubation of A.niger between concentration of 0.5% and 1% .Significance was between concentration 1% and 2%, 0.5% and 2% as shown in table(4).

DISCUSSION

In the present study, the peak of AOM detected at (4-6) years (55.2%) ; (7-9) years(24.14%) and (20.69%) for children (1-3)years. A.niger was isolated from children of (4-6) years (27.59%); followed by (1-3) years (17.24%) and in (10.34%) for children of (7-9) years old. Kids in the young age they are under continuous monitoring by parents, although the children at these ages are trying to discover the environment in which they live, especially in males, who are characterized by the love of discovery and hyperactivity than females and this agree with clinical findings of the present study in which male represent (69%), and female (31%). This may lead to the entry of foreign objects in the ear and that's where *A.niger* exist normally in the soil, and this may result in infection of middle ear. In addition the overall immune system in children weak and anatomy of the Eustachian tube where be short and straight /horizontal surface which helps to ease entry of pathogens from the nasopharynx, frequent upper respiratory tract infections also may facilitate A.niger associated AOM as a general stress factors for the children immune system[27,28,29,30].

Great variability in detection rate of A.niger was reported at different part around the word. The current study reflect high isolation rate for A.niger (55.2%) of AOM cases in children compared with other Iraqi studies indicating that (31.8 %) of patients less than 10 years and *A.niger* detected in 16.6%[31] while in Brazil 20% of Otomycosis caused by A.niger[32] and in Pakistan[33] the rate of ear infection by A. niger reach to (19.1%), in Nigeria, A.niger associated otitis media reported less than 0.48%[28].

Several Predisposing factors for A.niger associated otitis media such as a failure in the ear's defense mechanisms (changes in the coating epithelium, changes in pH, quantitative and qualitative changes in ear wax), bacterial infection, hearing aid or a hearing prosthesis, self-inflicted trauma (use of q-tips to clean the ear), swimming, broad spectrum antibiotic agents, steroids and cytostatic medication, neoplasia and immune disorders, all of which can render the children susceptible to the development of Otomycosis[32].

In current work, children suffering from AOM coming from poor family economy was(55.2 %) compared with (44.8%) good family economy. A.niger isolated from (17.24%) of infected children with good family economy compared with (37.93%) among those of Poor family economy. Children resident in old house represent (65.5%) compared with (34.5%) for new houses .A.niger was isolated from (34.48%) of children resident in old houses compared with (20.69%) positive culture for children resident in new houses. The majority of children (58.62%) presented with otitis media came from medium size families followed by those came from large families(27.59%). All cases of AOM for children came from large size families give positive culture for

Table 1: Demography of patients enrolled in current study.

Parameters			A.niger culture			Chi square	ANOVA*	
Age (years)	Minimum	1	Positive No.(%)	Negative No.(%)	Total No.(%)	P value		
	Maximum	9						
	Mean± SD	5.10 ± 1.97					F	P value
Age Groups (years)		1-3	10(17.24%)	2(3.45%)	12(20.69%)	**0.020**	4.463	0.039
		4-6	16(27.59%)	16(27.59%)	32(55.2%)	**1**		
		7-9	6(10.34%)	8(13.79%)	14(24.14%)	**0.593**		
		Total	32(55.2%)	26(44.8%)	58(100%)	**0.430**		
Gender	Male	40	22(37.93%)	18(31.03%)	40(69%)	0.004	**0**.001	0.969
	Female	18	10(17.24%)	8(13.79%)	18(31%)			
Economic Status	Good	26	10(17.24%)	16(27.58%)	26(44.8%)	0.431	5.656	0.021
	Poor	32	22(37.93%)	10(17.24%)	32(55.2%)			
Housing Status	Old	38	20(34.48%)	18(31.03%)	38(65.5%)	0.018	0.279	0.599
	New	20	12(20.69%)	8(13.79%)	20(34.5%)			
Family size	Small	<4	4(6.90%)	4(6.90%)	8(13.79%)	0.724	20.344	0.000
	Medium	(4–7)	12 (20.69%)	22(37.93%)	34(58.62%)	0.086		
	Large	>7	16(27.59%)	0(0%)	16(27.59%)	0.000		

*Differences between groups

Table 2: Distribution of infection according to moths of year.

Month	otitis media cases	*A.niger* culture		Chi square
	No.(%)	Positive (%)	Negative (%)	P value
January	2(3.4%)	2(3.4%)	0(0%)	0.000
February	0(0%)	0(0%)	0(0%)	
March	2(3.4%)	0(0%)	2(3.4%)	
April	2(3.4%)	0(0%)	2(3.4%)	
May	2(3.4%)	0(0%)	2(3.4%)	
June	4(6.9%)	4(6.9%)	0(0%)	
July	2(3.4%)	0(0%)	2(3.4%)	
August	0(0%)	0(0%)	0(0%)	
September	12(20.7%)	6(10.34%)	6(10.34%)	
October	12(20.7%)	10(17.24%)	2(3.4%)	
November	18(31%)	8(13.79%)	10(17.24%)	
December	2(3.4%)	2(3.4%)	0(0%)	
Total	58(100%)	32 (55.17%)	26(44.83%)	
Rho Correlation	Correlation Coefficient	0.093		
	P value	0.486		

A.niger(27.59%)with significant difference (p value =0.00006334) compared with (20.69%) for those from medium size families. only(6.90%) of otitis media cases give positive culture for those came from small size families. All these facts come in agreement and supported by other studies which indicating that *A.niger* associated AOM, commonly a disease of the developing world with malnutrition, over-crowding, substandard hygiene, frequent upper respiratory tract infections and under-resourced health care (all linked to low socio-economic status) listed as risk factors[29,30]. This study indicate a very important correlation between age of children, socio-economic status and overcrowding in large size families which constitutes a cardinal feature in rural areas in Iraq and the high isolation rate of A.niger. The poorer rural communities living in subsistence agricultural or slam areas have the highest prevalence[20,28,34,35]. Infection with A.niger detected more frequently in September ;October and November, while in February to May and in August, A.niger infection was not detected, this may reflect the environmental conditions which are favored by fungus for growth mainly temperature and humidity[36].

Alcohol extracts of *C.craniiformis* concentration (100mg, 200mg, 400mg, 500mg, 600mg, 800mg and 1000 mg) and ivermectin (0.5%,1 % and 2%) restricted the growth of *A niger* at 32- 35ºC on SDA after2-3 days of incubation (Figure 2, 4). While, no significant difference at 7th day of incubation and the growth of *A. niger* has spread to the surface area of the petri dish.

Significant difference in A.niger growth inhibition after 3 days of incubation between concentration 200mg and 400 mg;

400 mg and 600 mg; 800 mg and 1000 mg; 100mg and 1000 mg; 200 mg and 1000 mg. Meanwhile, no significant difference between concentration of 100 mg and 200 mg; 600mg and 800 mg. No significance difference in inhibitory activity of Ivermectin after 3 days of incubation of A.niger between concentration of 0.5% and 1%

Table 3: Correlations among sociodemographic factors and A.niger infection.

Parameters		Gender	Economy	House status	Month of infection	Number of Family members	A.niger
age	r	0.188	-0.048	-0.150	-0.114	-0.163	-0.272*
	P value	0.157	0.722	0.262	0.392	0.221	0.039
Gender	r		0.005	0.016	0.103	0.012	-0.005
	P value		0.969	0.904	0.440	0.926	0.969
Family	r			0.513**	0.071	-0.158	-0.303*
Economy	P value			0.000	0.594	0.238	0.021
House status	r				-0.150	0.256	0.070
	P value				0.260	0.052	0.599
Month of infection	r					0.141	0.108
	P value					0.292	0.422
Number of Family	r						0.516**
members	P value						0.000

* Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

Significance was between concentration 1% and 2%, 0.5% and 2% .

The medication used in this study is cheap, available, easily intake and safe. The present study disclosed that alcoholic extract of C.craniiformis (1000 mg) was effective in restriction of growth(22 ± 0.3mm) for A.niger AOM.. Aqueous solution of Ivermectin (2 %) was effective in restriction of growth(20 ± 1.3 mm) for A.niger associated AOM. In control group, fluconazole (150mg), the restriction for growth was (30 ± 1.3mm). *C.craniiformis* and Ivermectin appear to be not effective in current doses after 7 days. Which indicate the need for accumulative effect and stable concentration of agents under investigation. This is due to continues reduction in concentration of *C.craniiformis* and Ivermectin in SDA after 7 days, which become ineffective and resistible by A.niger.

Topically applied Ivermectin is absorbed by fungal cell and is actively transported across cell membranes by P-glycoprotein (P-gp).P-glycoprotein 1 (P-gp) also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) is an important protein of the cell membrane that pumps many foreign substances out of cells. It is an ATP- dependent efflux pump for xenobiotic compounds with broad substrate specificity[37]. It is responsible for decreased drug accumulation in multidrug-resistant cells. Substrate enters P-gp either from an opening within the inner leaflet of the membrane or from an opening at the cytoplasmic side of the protein[38] . Ivermectin has known as a member of the impressive list of inhibitors of P-gp-mediated transport. Ivermectin are able to interfere with P-gp transport activity. Because macrocyclic lactones are slowly transported by P-gp, it is suggested that when ivermectin binds to P-gp, the binding sites become unavailable to other compounds[38].

The possible cause of incomplete inhibition of A.niger, using aqueous solution of Ivermectin (2 %) because ivermectin is highly unstable in the presence of water and it is difficult to have stable pharmaceutical compositions . It exhibits the difficulty of being very sparingly soluble and rarely stable in water as a pharmaceutical solvent, and it is sensitive to an aqueous environment. However, ivermectin has very poor solubility in water, at a level of about 0.005 mg per ml at room temperature[39]. This sensitivity to water can result in chemical instability of the active principle and/or in crystallization of the initially dissolved active principle. This sensitivity to water thus limits its formulation in dermatological compositions administered via the topical route. The phenomena of chemical decomposition and/or of crystallization of ivermectin in the presence of water have as consequences a reduction in or loss of effectiveness and uncertainty with regard to the dose of active principle employed during the administration thereof, which militates against the desired objective[40]. Other factors may have a role in failure of treatment using aqueous solution of Ivermectin (2 %) is the requirement for more time for clinical cure and increase the concentration of Ivermectin for effective results.

The medical analysis of C.craniiformis proved the presence of three components; the first is calvatic acid , which has chemical formation P-carboxyphenyl-azoxycarbonitrile[41]. This calvatic acid proves a strong action against the yeast and fungi like Saccharomyces cerevisiae and several Candida species and Trichophyton asteroids[42]. The second components from chemical analysis and spectroscopic means of the mushroom powder is hydroxy phenyl azoformamide derivatives which has three chemical compounds ,4-hydroxyphenyl-1azoformamid, 4-hydroxyphenyl-ONN-azoformamid and 2-methylsulfonyl -4-hydroxy-6-methylthiophenyl-1-azoformamid, it is known craniformin (phenolic tautomer of rubroflavin), and also three components are known steroids, ergosta-4,6,8 (14), 22- tetraene- 3-one, ergosta-7,22-diene-3-01 and ergosterol peroxide[43]. The hydroxy phenyl azoformamide derivatives or craniformin have phenolics in its formation which are endowed with interesting biological activities as a broad spectrum bactericidal and fungicidal effect represented by Candida albicans and A niger[44]. The craniformin has azole compound which inhibits the synthesis of ergosterol by blocking the

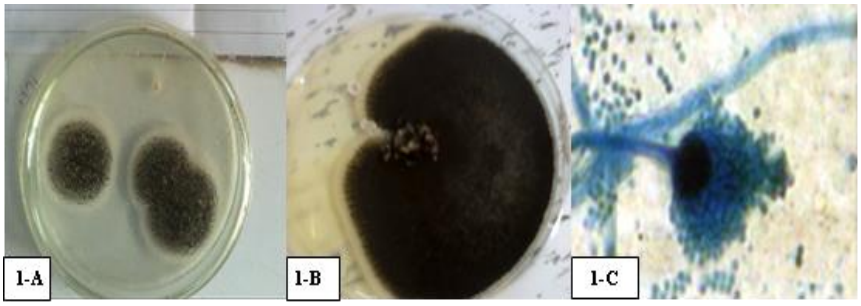

Figure 1: A: The primary growth of *Aspergillus niger* on sabauroud dextrose agar. B : The shape of purified isolate in sub culture. The center of the Petri dish appears white fluffy growth of colonies with elevated mycelia that turned black after 36 hours. (C): revealed microscopic character for the head of A. niger conidia.

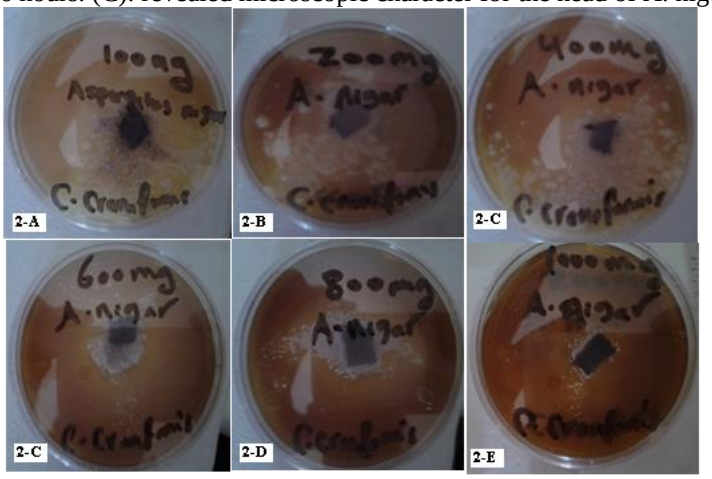

Figure 2: Different concentrations of C.craniiformis alcohol extract (100mg, 200mg, 400mg,600mg 800 mg and 1000 mg) against A. niger cultured on sabauroud dextrose agar after three days of incubation. (Restricted growth of A.niger).

Figure 3: Comparison of different concentrations of c craniiformis alcohol extract (100 mg, 200 mg, 400 mg, 600 mg, 800 mg and 1000 mg) and commercial antifungal (fluconazole) against A.niger cultured on sabauroud dextrose agar after seven days of incubation.(no restricted growth (diffusion all petri dish) of A.niger).

Figure 4: different concentrations of ivermectin (0.5%, 1% and 2%) against A.niger cultured on sabauroud dextrose agar after three days of incubation. (Restricted growth of A. niger).

Table 4: Antifungal Activity of *C. craniiformis* Alcoholic extract, ivermectin in Comparison with Fluconazole.

Antifungal agents	Concentration of Alcohol extract of *C. craniiformis*					
	100 mg	200 mg	400 mg	600 mg	800 mg	1000 mg
	Diameter of *A.niger* colony (mm) after 3 days of incubation					
	M ± SE	M ± SE	M ± SE	M ± SE	M ± SE	M ± SE
	34.5 ± 0.3	33 ± 0.3	31.5 ± 1.3	28.5±0.3	26 ±1.3	22 ± 0.3
	(a)	(*b)	(*c)	(*d)	(e)	(*,**,***f)
Alcohol extract of C *craniiformis*	Values : a, b, c, d, e, f, g ,h; significantly different level of P < 0.05 - a- No significance difference between concentration of 100 mg and 200 mg -*b- Significance difference between concentration 200mg and 400 mg -*c- Significance difference between concentration of 400 mg and 600 mg -e- No significance difference between concentration of 600mg and 800 mg -*f - Significance difference between concentration of 800 mg and 1000 mg -**f- Significance difference between concentration of 100mg and 1000 mg -***f- Significance difference between concentration of 200 mg and 1000 mg					
Ivermectin	Concentration of Ivermectin aqueous solution					
	0.5%		1%		2%	
	Diameter of *A.niger* colony (mm) after 3 days of incubation					
	M ± SE		M ± SE		M ± SE	
	26. ± 1.3 (a)		24.0 ±0.3(*b)		20 ±1.3 (*c)	
	- a- No significance difference between concentration of 0.5% and 1% -*b- Significance was between concentration 1% and 2% -*c- Significance difference between concentration of 0.5% and 2%					
Fluconazole	Concentration of Fluconazole 150 mg					
	Diameter of *A.niger* colony (M ± SE) in (mm) after 3 days of incubation					
	30.0 ± 1.3					

*Differences between groups

action of 14-alpha-demethylase and stop proliferation of the fungus[45]. The action of azole compounds reveals inhibition fungal mRNA transcription[46].

The chemical analysis of C.craniiformis powder which is done in white Fields Company for chemical and engineering studies and consultations in Baghdad – Iraq proved the presence of different materials as gallic acid and others. Gallic acid is a trihydroxybenzoic acid, a type of phenolic acid and found both free and as a part of tannins. Gallic acid seems to have anti-fungal properties[47]. The oral fluconazole used in current study is a tri azole antifungal agent acts by inhibiting cytochrome P450-dependent ergosterol synthesis in fungal cells in a similar manner of itraconazole and ketoconazole[48]. The efficacy of oral fluconazole reported in the present study comes closely to[49], in which four weeks after the last treatment, the mycological cure using 150 mg fluconazole was (73%). Oral fluconazole therapy was found to be superior than other topical remedies like clotrimazole in the treatment of pityriasis versicolor in terms of efficacy and patient compliance and also cost-effective for the patients[50], but the efficacy depends on increasing of dose up to 300 mg weekly to be more potent, which is the main drawback due to possible toxicological effects[48,50].

In conclusion, otitis media associated A.niger infections represent a clinical problem in children and need serious attention from clinicians. The present study notes that there is a combination of factors can play a fundamental role in reducing the infection middle ear due to fungi. The first of these factors improve the living conditions of the people in the village and improve the level of education areas. Improve municipal services and attention to the cleanliness of the environment in rural areas .attention for building new homes relatively wide and make families less crowded. A.niger infections have positive correlation with family size and inverse correlation with patients age and family economy .No correlation between A.niger and month of the year. The growth of A niger significantly restricted by alcohol extract of C.craniiformis and Ivermectin in concentration dependent manner. The powerful concentration was 1000mg.for C.craniiformis and 2% for Ivermectin. Thus, *C.craniiformis* and Ivermectin represent a novel antifungal agents that can be used in clinical practice for treatment of A.niger associated otitis media that represents a clinical problem in children and need serious attention from clinicians.

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