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ORIGINAL RESEARCH ARTICLE

Effect of Ethiopian multiflora honey on fluconazole-resistant *Candida* species isolated from the oral cavity of AIDS patients

A Mulu BSc MSc PhD[*], **E Diro** MD[†], **H Tekleselassie** MD MSc[‡], **Y Belyhun** BSc MSc[*], **B Anagaw** BSc MSc[*], **M Alemayehu** BSc MSc[*], **A Gelaw** BSc MSc[*], **F Biadglegne** BSc MSc[*], **K Desalegn** MD[§], **S Yifiru** MD[**], **M Tiruneh** BSc MSc PhD[*], **A Kassu** BSc MSc PhD[*], **T Nishikawa** MD PhD[††] **and E Isogai** DVM PhD[‡‡]

[*]Department of Microbiology and Parasitology; [†]Department of Internal Medicine, College of Medicine and Health Sciences, University of Gondar, Gondar; [‡]Department of Surgery, Facility of Medicine, Addis Ababa University, Addis Ababa; [§]Department of Dermatology; [**]Department of Pediatrics and Child Health, College of Medicine and Health Sciences, University of Gondar, Gondar, Ethiopia; [††]Hokkaido University of Education, Division of Medicine and Nursing, Sapporo; [‡‡]Department of Disease Control and Molecular Epidemiology, Health Sciences University of Hokkaido, Ishikari-Tobetsu, Hokkaido, Japan

Summary: This study aimed to determine the antifungal effect of Ethiopian multiflora honey against *Candida* species isolated from the oral cavity of AIDS patients. Oral rinses were obtained from 13 AIDS patients and cultured on CHROMagar plates at 37°C for 48 hours. *Candida* species were identified by microbiological and molecular techniques. The antifungal effect of the honey sample on *Candida* was investigated by an agar dilution technique. Susceptibility of the *Candida* species to fluconazole was tested following a semi-modified microdilution method. Growth of both fluconazole-susceptible and -resistant *Candida* species was inhibited with a minimum fungicidal concentration (MFC) of 35–40% (v/v) honey. The MFC of different *Candida* species was not significantly different ($P > 0.05$). From the total of 25 *Candida* isolates tested for susceptibility, 11 (44%), eight (32%) and six (24%) of the isolates were sensitive (minimum inhibitory concentrations [MICs] < 8 μg/mL), susceptible (dose-dependent: MICs 16–32 μg/mL) and resistant (MICs > 64 μg/mL) to fluconazole, respectively. Ethiopian multiflora honey has antifungal activity against fluconazole-resistant *Candida* species isolated from the oral cavity of AIDS patients. This supports the existing folkloric practice of using honey to treat oral lesions. Nevertheless, identification of the bioactive agents in honey, their clinical evaluation and pharmacological standardization are crucial.

Keywords: candidiasis, HIV/AIDS, antifungal effect, honey

INTRODUCTION

Infection with human immunodeficiency virus (HIV) and the resulting acquired immunodeficiency syndrome (AIDS) continues to be the major health problem throughout the world. According to the World Health Organization (WHO), more than 22.5 million people were living with HIV/AIDS in sub-Saharan Africa by the end of 2007. In Ethiopia, the epidemic, as in the other sub-Saharan African countries, has been spreading rapidly with an estimated adult prevalence of 2.1%.[1]

Infections with *Candida* species and other fungi have increased dramatically in recent years, and are of particular importance because of the rising number of immunocompromised patients due to HIV/AIDS.[2] Oropharyngeal candidiasis (OPC) is the most common opportunistic fungal infection in HIV-positive individuals.[3–5] A vast majority of HIV-positive patients develop clinical lesions of OPC or esophageal candidiasis due to *Candida albicans* that can increase in frequency and severity with HIV disease progression.[6] However, during the last 20 years a marked shift in the spectrum of *Candida* species (such as *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis* and *Candida krusei*) has been noted among different immunocompromised patients with CD4 lymphocyte counts less than 200 cells/mm^3. These *Candida* species are of increasing significance as they tend to be more resistant to antifungal agents.[7–9] The pattern of *Candida* species in Ethiopian HIV/AIDS patients has not been well described except for a single study conducted over a decade ago.[10]

The introduction of highly active antiretroviral therapy (HAART) has dramatically reduced the incidence of opportunistic infections among HIV-positive people who have received the drugs.[11] A marked decrease in incidence of oral lesions has been reported in patients receiving HAART, related to the improvement in immunological function or to inhibition of the fungal secretory aspartyl proteinases that play a pathogenic role in mucosal invasion.[11–13] However, millions of people living with HIV in resource-poor settings have no or limited access to HAART. Due to this, particularly in rural areas, patients prefer to visit traditional healers. In addition, despite the enormous advances made in health care during the last half-century, the effectiveness of antimicrobial drugs, including antifungals, is reducing as resistant microbes develop and spread.[14] This highlights the need to look for alternative treatment options. In response, the WHO has adopted a

Correspondence to: A Mulu
Email: andargachewmulu@yahoo.com

revolutionary approach of exploring ways in which scientific medicine and traditional medicine can work together to solve the world's health problems.[15]

Honey has been reported to have an inhibitory effect on around 60 species of bacteria including aerobes and anaerobes, Gram positives and Gram negatives,[16] and yet data on antifungal activity of honey remain limited. However, antifungal action has been reported against some species of *Aspergillus* and *Penicillium* as well as on the common dermatophytes.[17–19] Despite the advent of antibiotics, honey has continued to play a major role in folk medicine. It is from this pool of knowledge that the reintroduction of honey into modern medicine has emerged. There have been numerous reports in scientific literature about the use of honey in folk remedies as a last resort on infected wounds, burns and ulcers that were not responding to antibiotic treatment.[16–21] However, its antifungal activity in general and its anticandidal activity in particular have not been well described. The present study was intended to determine the antifungal effect of Ethiopian multiflora honey produced by *Apis melifera* against *Candida* species isolated from the oral cavity of HIV/AIDS patients.

METHODS

Sample collection, isolation and identification of *Candida*

HIV seropositive subjects who visited the Black Lion Specialized Hospital, Addis Ababa, Ethiopia in May 2007 with different clinical stages were randomly selected. After informed and written consent was obtained, the study subjects were instructed to provide a rinse of their oral cavity. Briefly, oral rinses were obtained from each subject by asking them to rinse their mouth with 5 mL of sterile normal saline (0.85% NaCl solution) for 30 seconds and to expectorate the rinse into a sterile container. Then, the entire fluid was poured onto a CHROMagar plate (CHROMagar, Paris, France), a selective medium for isolating *Candida* species, which was prepared as per the manufacturer's instructions. After 30 seconds, the plates were drained and incubated at 37°C for 48 hours.[22] Primary identification of *Candida* was made based on colour of the colonies as per the protocol of the manufacturer as *C. albicans* (light mint green), *C. tropicalis* (steel blue to bluish purple) and *C. glabrata* (red purple). Further identification of *Candida* was done using tobacco agar. Moreover, DNA was extracted from the colonies and the rRNA gene was amplified by polymerase chain reaction (PCR) using species-specific primers. Restriction fragment length polymorphism and sequence analyses of the amplicons were performed following standard protocols as described previously.[23] Isolates were stored at −40°C and cultured on CHROMagar at 37°C prior to being tested for their susceptibility.

Preparation of honey and the media

A multiflora honey sample from the Amhara region of Northwest Ethiopia, where different kinds of flowering plants grow naturally, was obtained between October and December in 2007. The honey sample was first streaked on a blood agar plate, and incubated at 37°C overnight to check for microbial purity and then stored at 2–8°C until used (June–July 2008). The honey sample used in this study had antibacterial activity as reported by our previous works.[20,21] A known volume of molten CHROMagar was held in a water bath (45–50°C) and mixed with a known volume of honey to get an equivalent concentration of honey constituting 5, 10, 15, 20, 25, 30, 35 and 40% (v/v) to give a total volume of 20 mL preparation.[24]

Determination of minimum inhibitory concentration (MIC)

Determination of the MICs of honey on 25 randomly selected *Candida* species: 10 *C. albicans*, 10 *C. tropicalis* each isolated from 10 different patients and five *C. glabrata* isolated from two different patients was carried out using the agar dilution technique following the standard procedure. The 25 isolates were subcultured in CHROMagar plate to produce a fresh growth. From the fresh colonies, a turbidity that matches to 0.5 McFarland standards (1×10^6–5×10^6 cells/mL) was prepared with sterile normal saline. A working suspension was made by a 1:100 dilutions of the stock suspension resulting in 1×10^4–5×10^4 cells/mL. Within 10 minutes of inoculum standardization, microbial pure mediums (containing different concentrations of honey) were inoculated with a calibrated loop full (0.01 mL) of the prepared fungal cultures using the spread plate technique and incubated aerobically at 37°C for 48 hours in inverted positions. CHROMagar plates without honey were also used similarly to provide appropriate growth control.[22]

Complete inhibitory effect of different concentrations of honey was best examined by placing plates on a dark background and observing macroscopically using a magnifying lens for the lowest concentration that completely inhibited growth in comparison with the control plates. Thus, the MIC of honey was reported as the lowest concentration of honey that completely inhibited visible growth. Furthermore, the last plate which showed visible growth and all the plates in which there were no growth were swabbed with a moisturized cotton wool swab and cultured on CHROMagar plate without honey. The lowest concentration of honey required to produce a sterile culture was therefore taken as the fungicidal concentration of honey.[24] A supersaturated solution of sugar (sucrose) of the same sugar proportion as in honey (85% w/v) and an autoclaved honey sample of the same type as used above were used on *C. albicans* for comparison purposes following the same procedure. This test was performed once in triplicate and the average value ± SD was taken. *P* values were calculated using SPSS Statistical Software version 13.0.

Susceptibility of *Candida* species to fluconazole was tested as described by Anaissie *et al.*[25] and Margaret *et al.*[26] with a slight modification. Briefly, inoculum suspension was adjusted to a 0.5 McFarland Standard to produce 1×10^6–5×10^6 cells/mL. A working suspension was made as by a 1:100 dilutions. Immediately, 10 μL of this saline suspension was transferred to 10 mL of sterile saline. After the suspension was mixed, it was poured into an inoculation tray. By use of a 12-channel pipette and sterile tips, 10 μL of the inoculum was added to microtiter plate wells 1–11 (wells 1–10 contained 0.125 to 64 μg of fluconazole per mL; well 11 was the growth control without the fluconazole); well 12 was the sterility control or blank in Tryptose Soya Broth supplemented with 0.5% yeast extract.[26] This inoculum is equivalent to 5.0×10^2–2.5×10^3 cells/mL. The microtiter plate was shaken on a plate shaker for 30 seconds to ensure even distribution of the inoculum. After incubation at 37°C for 48 hours, the microtiter plate

Table 1 **Antifungal activity of Ethiopian multiflora honey, fluconazole and 85% (w/v) sucrose solution**

Characteristics	Average anticandidal activity	*Candida* species		
		C. albicans	*C. tropicalis*	*C. glabrata*
Untreated honey	MIC% (V/V)	40.0 ± 2.6	35.0 ± 3.4	35.0 ± 3.7
Autoclaved honey	MIC% (V/V)	45.0 ± 3.3	39.5 ± 1.6	39.5 ± 2.2
Fluconazole	MIC (μg/mL)	6. 6	5.8	5.2
85% (w/v) Sucrose	MIC% (V/V)	NI*	NI*	NI*

*No inhibition up to 85% (w/v)

was taken out from the incubator and shaken for five minutes, and the optical density of each well was read at 530 nm. The MIC was considered to be that concentration of drug which allowed 25% or less growth than that of the control.[25–27] Breakpoint definitions for fluconazole were based on NCCLS M27 A, and were as follows: sensitive MIC < 8 μg/mL, susceptible-dose-dependent (S-DD) MIC = 16–32 μg/mL and resistant MIC > 64 μg/mL.[27]

This study was approved by the University of Gondar Institutional Review Board. After the goals and objectives of the study were explained, informed consent was obtained from each study participant. Absolute confidentiality was maintained.

RESULTS

Candida species were isolated from oral rinses of all 13 patients and a total of 71 colonies were yielded. The dominant species observed was *C. albicans* (41/71) followed by *C. tropicalis* (15/71), *C. glabrata* (9/71), *C. dubliniensis* (2/71), *C. kefyr* and *C. krusei* (2/71). As shown in Table 1, the growth of all *Candida* species was largely inhibited by honey. The MIC of honey for all *Candida* species ranged from 35 to 40% and was not significantly different ($P > 0.05$). Partial inhibition (colony reduction) for all *Candida* species was observed starting from 5% (v/v) honey. Complete inhibition was observed at 35% (v/v) of honey for *C. albicans* and 40% (v/v) for the rest of the *Candida* species tested. Subculturing after determining the MIC showed that growth of *Candida* species occurred when they were subcultured from plates with concentrations of honey below the MIC, whereas there was no growth from concentrations at and above the MIC. This revealed that honey has a killing effect for all of the *Candida* isolates in this study. A significant dose-dependent colony reduction has been observed as low as 5% honey concentration (Figure 1). The MIC_{50} of honey for *C. albicans*, *C. tropicalis* and *C. glabrata* were found to be 20–25%, 15% and 15–20%, respectively. The MIC_{90} was 35–40% for *C. albicans* and 25–30% for *C. tropicalis* and 30–35% *for C. glabrata.*

This study also assessed the anticandidal activity of honey after autoclaving the honey sample at 121°C for 15 minutes. The anticandidal activity of honey was decreased by 4.5–5% after heat treatment and the difference, compared with the heat-untreated honey, was not statistically significant ($P > 0.05$). The anticandidal activity of honey was also compared with a supersaturated solution of sugar of the same sugar proportion as in honey (85% w/v). The supersaturated solution of sugar did not exhibit anticandidal activity (Table 1).

The MIC of fluconazole was summarized in Table 2. From the total of 25 *Candida* isolates, 11 (44%), 8 (32%) and 6 (24%) of the isolates were sensitive (MICs < 8 μg/mL), S-DD (MICs

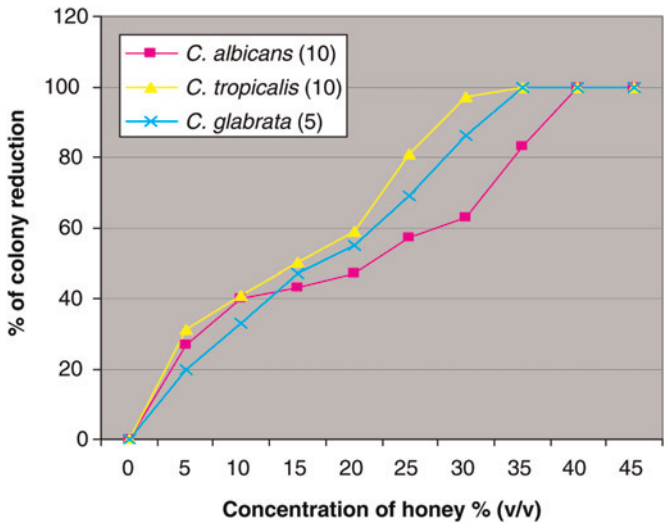

Figure 1 **Dose-dependent anticandidal effects of Ethiopian multiflora honey on *Candida* species**

16–32 μg/mL) and resistant (MICs > 64 μg/mL), respectively. The mean MIC value was found to be 6.6 μg/mL, 5.8 μg/mL and 5.24 μg/mL for *C. albicans, C. tropicalis* and *C. glabrata*, respectively. *Candida* species which were resistant to fluconazole were inhibited with 35–40% (V/V) honey concentration.

DISCUSSION

OPC is an opportunistic fungal infection which can be acute or recurrent. It is common in immunocompromised patients, especially those infected with HIV, and is often one of the first clinical signs of underlying HIV infection. OPC is manifested by 50 to 95% of all HIV-positive persons at some time during their progression to full-blown AIDS.[28,29]

Table 2 **Fluconazole susceptibility profiles of *Candida* species isolated from HIV/AIDS patients**

Candida isolates (number)	Fluconazole susceptibility		
	Sensitive (MIC ≤ 8 μg/mL)	S-DD* (MIC = 16–32 μg/mL)	Resistant (MIC ≥ 64 μg/mL)
C. albicans (10)	3 (30%)	4 (40%)	3 (30%)
C. tropicalis (10)	5 (50%)	3 (30%)	2 (20%)
C. glabrata (5)	3 (60%)	1 (20%)	1 (20%)
Total isolates (25)	11 (44%)	8 (32%)	6 (24%)

*Susceptible-dose dependent

The increase in drug-resistant microbial infections highlights the need to look for alternative treatment options. Honey, one of the oldest traditional medicines, is being used effectively as a dressing for wounds, burns and skin ulcers. It completely inhibits the growth of Gram-positive and Gram-negative bacteria.[16,17,19,20,21] Although the honey sample used in the present study showed antibacterial activity,[20,21] its antifungal activity had not been elucidated. Interestingly, the present study revealed that the growth of *Candida* species was largely inhibited with MIC value of 35–40% (v/v). This supports the existing folkloric practice of using honey to treat oral lesions.[20,21] For all *Candida* species, the MIC of the honey sample was not significantly different ($P > 0.01$). The MIC of honey was 40% for *C. albicans* and 35% for *C. tropicalis* and *C. glabrata*. Similar MIC values have been reported by Irish *et al.*[30] and Koc *et al.*[31] However, the MIC of honey in the present study was found to be lower as compared with a report from Turkey where the antifungal activity of honey was found to be 80% (v/v).[31] This variation in the antifungal activity of honey highlights that the source of the nectars (the flowers from which bees gathered nectar to produce the honey) may have contributed to the difference in the antimicrobial activities of honey, since flora source determines many of the attributes of honey.[32]

It has been demonstrated in many studies that honey has antibacterial effects, attributed to its high osmolarity, low pH, hydrogen peroxide content and content of other uncharacterized compounds such as tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, flavonides, streptomycin, sulfathiazole, terpenes, benzyl alcohol and benzoic acids.[19,33–35] This study compared the antifungal activity of honey to a supersaturated solution of sugar of the same sugar proportion as in honey [85% (w/v) sucrose solution], but this showed no degree of antifungal activity, indicating that while the removal of water from microorganisms is important, other factors are responsible for the observed antifungal effect, which is in agreement with previous reports by Molan.[19,33]

Our findings showed that the antimicrobial substance in honey could withstand heating at 121°C for 15 minutes. This also suggests that the antimicrobial activity of honey is not dependent only on its phytochemical nature, since tetracycline derivatives, ascorbic acid, peroxidase, amylases, streptomycin and sulfonamides are claimed to be heat labile.[36] In contrast, the antimicrobial effect of honey is attributed to its phenolic acid, flavonides and benzyl-alcohol, 2-hydroxy benzoic acids which are heat stable and may be the active agents although their concentration in honey appears to be very low.[32] However, there was an initial report as cited by Molan[19] on the loss of antibacterial activity of honey on exposure to heat, reported as 17–50% when honey was exposed to 56–100°C for 5–30 minutes. While Molan demonstrated reduction in the antimicrobial effectiveness of honey with heat, there was no significant reduction in its efficacy in our study. This may result from the difference in the honey type. Molan also cited another report from Germany that exposure of honey to 100°C for 5 minutes or 56°C for one hour caused complete loss of inhibition by 17% of honey.[19] Many workers have demonstrated that not all honey samples have the same degree of antibacterial activity. Therefore, the sensitivity of *Candida* cannot be compared using the results from different studies, as the honeys used in the studies may have had widely differing antimicrobial activity. The sensitivity of species relative to each other can be validly determined within a single study in which the same honey and same test conditions are used.

Fluconazole is considered the drug of choice for treating oral candidiasis in HIV/AIDS patients[34] and emergence of fluconazole-resistant *Candida* species is seen with secondary and prolonged exposure to fluconazole.[35] Currently, advanced immunosuppression[37] is also major risk factor for fluconazole resistance. Our findings showed that 30% (3/10) of *C. albicans*, 50% (5/10) of *C. tropicalis* and 60% (3/5) of *C. glabrata* were susceptible to fluconazole and 24% (6/25) of the total isolates showed *in vitro* resistance to fluconazole. This is in agreement with a study from Nigeria.[38] The susceptibility profile of the sensitive *Candida* organisms showed activity at relatively high MICs (mean MIC 5.86 μg/mL). Our study showed that *C. albicans* generated relatively high-fluconazole MICs (6.6 μg/mL) compared with *C. glabrata*, which has been reported to show high-fluconazole MICs.[39] This does not, however, prove that fluconazole would still be efficacious in the management of candidal infections in HIV/AIDS patients from this locality and this warrants consideration in management of recurrent oral candidiasis. Interestingly, this study demonstrated that honey has a significant antifungal effect among fluconazole-resistant *Candida* species.

In conclusion, honey has a significant antifungal activity on *Candida* species, some of which were resistant to fluconazole when tested *in vitro*. This supports the existing folkloric practice of using honey to treat oral lesions. Nevertheless, identification of the bioactive agents in honey with antifungal effects, and their clinical evaluation and pharmacological standardization remain key.

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