

***In vivo* and *In vitro* studies of the inhibitory effect of *Anethum graveolens* essential oil on *Candida albicans* growth and infection**

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Abstract: *Candida albicans* is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans. Until now, there is no active and safe antifungal agent. The medicinal plant *Anethum graveolens* (Dill) was collected and essential oil (E.O) was extracted using soxhlet. The antimicrobial activity of the obtained oil was determined against *C. albicans* as test organism using agar well diffusion method. The extracted oil showed excellent activity against *C. albicans* with MIC of 0.0312 µg/ml. In time–kill experiment, the log time–kill was 3.08. Immunosuppressed mice was infected with *C. albicans* and treated with *A. graveolens* E.O. The treated mice had a significantly lower number of the collected *Candida* (cells/swap) compared to untreated mice (control). Comparative Histological studies of epithelium of dorsal tongues stand with PAS stain confirmed the anticandida activity of the oil and there is a reduce number of *C. albicans* cells in epithelium layers of tongue after treatment with *A. graveolens* E.O. In conclusion, essential oil of *Anethum graveolens* can be used to treat fungal infection.

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1. Introduction

Anethum graveolens (Dill), is a short-lived perennial herb, oval fruit about a tenth of an inch wide (Jana and Shekhawat, 2010 and Khare, 2004) and find natively in southwest of Asia and Europe. *Anethum. graveolens* has been reported to contain flavonoids, phenolic, and essential oil (Delaquis *et al.*, 2002) which is extracted from the seeds, leaves, and stems of the plant (Jana and Shekhawat, 2010 and Ravindran and Balachandran, 2005). The chemical components of essential oil from the seed of *A. graveolens* are carvone and limonene which possess various degrees of antimycobacterial activity and to exhibit strong antifungal activity (Delaquis *et al.*, 2002 and Arora and, Kaur, 2007) and some researchers have reported that essential oil from the seed of *A. graveolens* possessed anti- *Candida* (Yili *et al.*, 2009 and Jirovetz *et al.*, 2009). *Candida albicans* is a normal component of the oral flora and only produces disease under unusual circumstances. Fungal infection (mycosis) of any *Candida* species, of which *C. albicans* is the most common, also referred to as yeast infection called Candidiasis (William *et al.*, 2006) which is an acute or chronic skin and mucous membrane infections, causing serious systemic disease (Bhattacharyya *et al.*, 2003).

In immunosuppressed or debilitated individuals, such as diabetics, the infection usually remains superficial, but can spread to deep sites in association with more severe immunosuppression, including that

seen in organ or hematopoietic stem cell transplant recipients, as well as patients with neutropenia, AIDS, chemotherapy induced immunosuppression and in newborn where colonization usually occurs from the mother's vaginal flora or other exogenous sources (Kumar *et al.*, 2013, Maenza and Merz, 1998). In this study, immunosuppression mice model was used to study the effect of oil plant extract as an antifungal agent to decreased candidal growth *in Vivo*.

2. Material and Methods

Plants collection and oil extraction

Anethum graveolens seed were purchased from the local market of Jeddah, and identified at Plant Department, Faculty of Science, KAU. Essential oil of *Anethum graveolens* was prepared as described by Ashraf *et al.*(2011)

Determination of anticandidal activity

Candida albicans ATCC66027 was obtained from Microbiology laboratory, KAU Hospital. The anticandidal activity of *Anethum graveolens* E.O on *C. albicans* *in vitro* was determined by agar well diffusion method of (Perez *et al.* 1990) with some modified. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *Anethum graveolens* E.O on candidal growth were determined using Micro broth dilution assay according to CLSI method (Rex *et al.* 1997).

Time-kill assay

According to the method described by (Klepser *et al.* 1998 and Quan *et al.* 2006) time-kill curves were performed by using 20 ml of Sabouraud dextrose containing 10^7 cfu/ml of *C. Albicans* and deferent concentrations of *A. graveolens* E.O. *Candida* growth was determined after 0, 4, 8, 12, 24 and 48 h of incubation.

In Vivo effect of *Anethum graveolens* E.O on *C. albicans* in immunosuppressed model

Thirty female mice, 6 week old, weighing 25–30 gm each, were obtained from the Animal House, KFMR and kept under controlled temperature ($22\pm 2^\circ\text{C}$), humidity ($55\pm 10\%$), and 12/12 hours cycle of light and dark with an access to food and drinking water ad libitum. The animals were immunosuppressed and treated with Takakura method (Takakura *et al.* 2004) and arranged into 3 groups; first group was control group which was resaved physiological solution. Second group was infected group which was infected with *Candida* cells and wasn't resaved any treated. The third group was treatment group with *A. graveolens* oil suspended in 1% Tween 80 and pitted onto the mice oral cavity.

Histological analysis

For histological analysis the tongue were fixed in 10% formalin for fixation, after embedding in paraffin. $5\mu\text{m}$ thick slices of tissue were cut and stained with Periodic acid-Schiff PAS stain. The presence of *C. albicans* was determined by using light microscopy at magnification of 200X. The number of *C. albicans* cells in oral cavity post and after treatment was counting by swapped a cotton swab in oral cavity then cut off the end off swab in 5 ml of the sterile saline solution and serial dilution were prepared. About 0.1ml of 10^{-2} sterile dilution was culture in Sabouraud dextrose agar for 24h at 37°C .

Statistical analysis

The data of the \log_{10} CFU of *C. albicans* using a Student's t test, P value of < 0.005 were considered significant result. The data contain the mean values were include the standard deviation of the mean.

3. Results

Effect of *A. graveolens* E.O on *Candida albicans* growth, in Vitro study

Anethum graveolens E.O showed inhibitory effect on *C. albicans* growth using well diffusion method with mean 18.3 ± 1.5 mm diameter of inhibition zone (Figure 1).

The CLSI test showed efficacy of *A. graveolens* E.O on *C. albicans* growth and the MIC and MFC value was calculated to be $0.0312\ \mu\text{g/ml}$. In time-kill

experiment, the log time-kill was 3.08 which is highly significant at $P= 0.001$ (Figure 2).



Figure 1. Zones of inhibition produced by *A. graveolens* EO using agar well diffusion assay and *C. albicans* as test organism

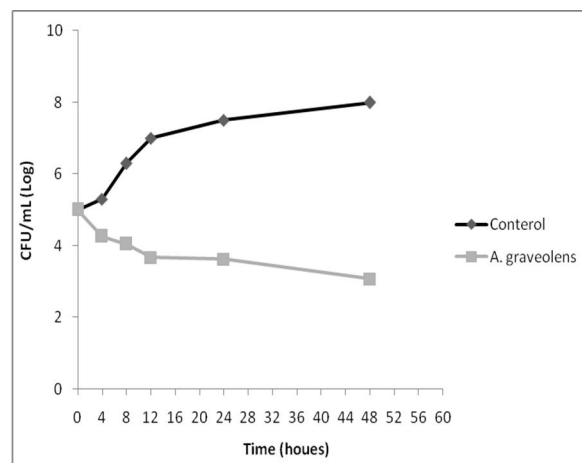


Figure 2. Representative time-kill curves for *C. albicans* and 1MIC of *A. graveolens* EO.

Effect of *A. graveolens* E.O on *C. albicans* in immunosuppressed model

Experimental animal model was used to assessing *A. graveolens* E. O efficacy against *C. albicans* in Vivo. The treated group was responded to *A. graveolens* E.O therapy with decreasing the number of the collected *Candida* cells/swab and the mean log CFU/swab of *C. albicans* was 2.6 which was highly significant at $P < 0.001$ (Figure 3) compared to infected group. Comparative histological studies of epithelium of dorsal tongues stained with PAS stain in both groups showed highly degree of candidal invasion and cells penetrated keratin layer in control group which had flat surface of epithelium and abnormal papillae. There were a

reduced number of *C. albicans* cells in epithelium layers of tongue after treated with *A. graveolens* E.O (Figure 4).

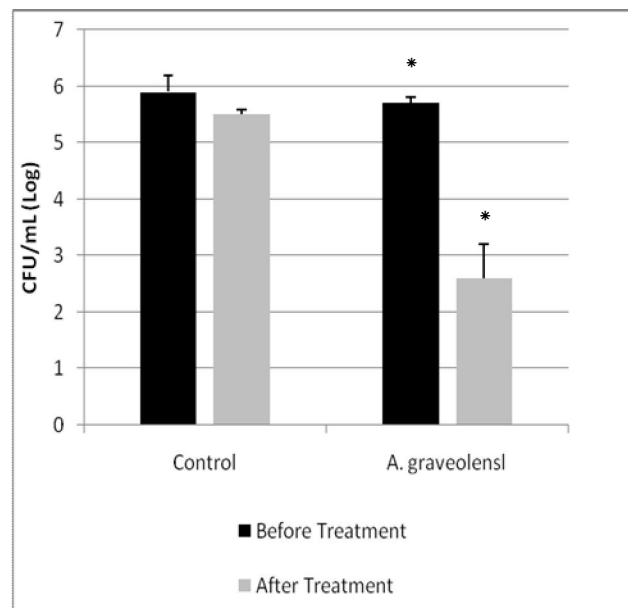


Figure 3. Number of *C. albicans* cells in oral swab post and after treatment with *A. graveolens* E.O. *highly significant difference between CFU/ml post and after treatment at $P < 0.001$

4. Discussions

Anethum graveolens is an important member of the family Umbelliferae and natively found in southwest of Asia and Europe and is widely used for flavoring foods, beverages and for the treatment of many pathological conditions and diseases (Chen et al 2013). Resent study showed great antifungal activity of dill essential oil obtained using hexane as a solvent or hexane and acetone polar nature, due to their volatility, miscibility with polar and non polar solvents and relatively lower toxicity (Eloff 1998). Dill EO inhibited of fungal growth, fungal adherence to the plastic substratum (Grumezescu et al. 2011). According to the present study, the *A graveolens* EO proved to have a strong inhibitory effect on *C. albicans* infections in vitro that result in effective proven in killing time curve assay. Concerning immunosuppressed control group, the examined sections remained infected with epithelial damage during the excrement time, while the treatment rats showed high significant of healing under light microscopic examination.

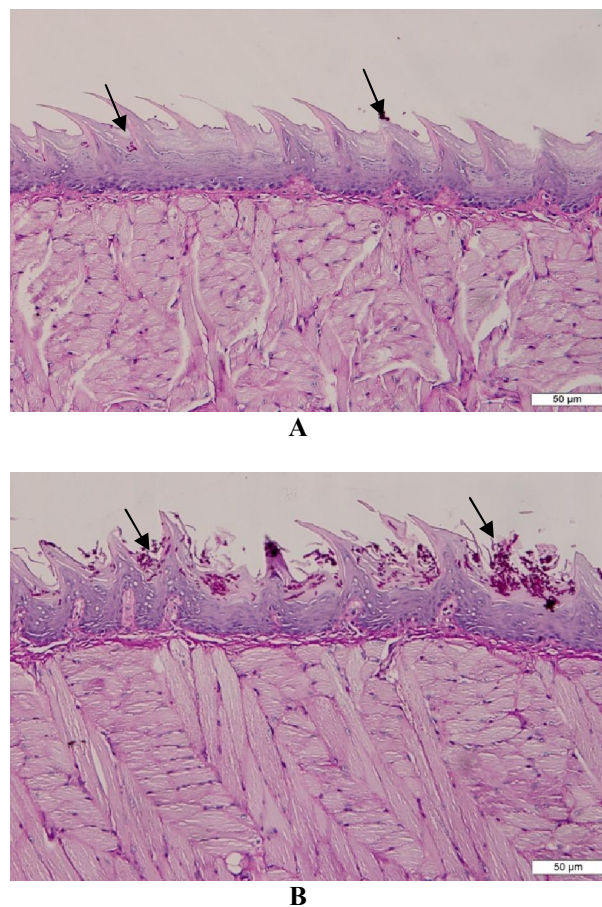


Figure 4. Sagittal section of lingual mucosa of candidal infection longue stained with PAS stain at magnification of 400 X. A: Control group showed *Candida* invasion and penetration of keratin layer by fungal hyphae. B: Treated group with *A. graveolens* E.O, showed a reduced number of *C. albicans* cells.

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