The anti-fungal effect of flax seed on oral candidiasis: Comparative In-Vitro Study

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Abstract

Flaxseed is a natural product with a wide array of anticancer, antibacterial, antiviral and also antifungal properties. Flaxseed extracts; either oil or water based can be incorporated into pharmaceutical compounds to be used in clinical setting. This study aims to compare the in-vitro antifungal effectiveness of flaxseed extract against the commonly used synthetic compound, Nystatin. Methodically, antifungal effectiveness of flaxseed extract and Nystatin was tested upon the *Candida albicans* culture growth in petri dishes. Disc diffusion method was performed and the zones of inhibition around the disc within each petri dish were measured after 48 hours of incubation period. Oil-based and water-based flaxseed extract types were evaluated using disc diffusion method at different volume per disc (5µl, 10µl and 15µl) and its result was compared to Nystatin's effectiveness. The principle result shows that the oil-based extract exhibited no antifungal activity despite of the increasing quantity used. Interestingly the comparison of mean value for candidal growth inhibition diameter between flaxseed aqueous extract and Nystatin was significant based on p-value less than 0.05. Clearly, *Candida albicans* growth diameter mean value was greater with higher increment of flaxseed aqueous extract when compared to the Nystatin 100 units control test. The greatest zone of inhibition was seen with 15µl of flaxseed aqueous extract. Thus, water-based flaxseed extract has a great potential to be used as a clinical product to control oral fungal infection while eliminating the unwanted side effects commonly occurring with synthetic products.

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Introduction

In 1665, Candidiasis resulted by *Candida albicans* was considered to be fatal disease with no viable countermeasures. It was not until 1949 when Nystatin was discovered and later similar antifungal such as Amphotericin B that this condition was brought under control. [1]Currently, the most widely used drug for treating candidiasis is fluconazole, a triazole drug. However so, the developmental process of antifungal drugs took decades despite having the knowledge of their existence.[2] Fungal infections are able to thrive well mainly due to the fact that the eukaryotic species biochemistry is similarly comparable to that of a human host. It is this factor that has made synthesizing a drug to combat fungal infections whilst leaving the human host unaffected proven to be very difficult.[3]It is henceforth why most of the currently available antifungal drugs are known to cause side effects whereby some of the side effects can be life-threatening namely, if the drug is misused. [4]Some of the side effects caused by fungicide troches, pastilles and even gargle solution include altered taste, stomach discomfort, oral irritation, nausea, vomiting, diarrhea, headache, dizziness loss of hair, anaphylactic reactions,

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adrenal insufficiency, hepatoxicity as well as nephrotoxicity.[5]

In fact, report regarding adverse or undesirable side effects as well as an increase in resistance of the fungal strain against synthetically produced antifungal drug was recorded since its inception from the early 1950's. While numerous research and modification has been made on the synthetic form of Nystatin as well other commercially available antifungal agents, it is still far from being devoid of side-effects. [6]Furthermore, the past decade has bear witness to a drastic hike in the resistance of fungal microbes towards existing antifungal agent.[7] A recent study have expounded the fact that clinical resistance of *Candidaalbicans* towards fluconazole which is a relatively new antifungal drug is on the rise, [8]especially so in HIV afflicted population.[9]Usage of herbal compounds can provide a viable solution to these issues. One such herb that is being widely being spoken of is flaxseed.

Flax; also known as common flax or linseed (binomial name: *Linum usitatissimum*) is a member of the genus *Linum* in the family Linaceae. It is native to the region extending from the eastern Mediterranean to India and was probably first domesticated in the Fertile Crescent. Known by many names it is termed as "Agasi/Akshi" in Kannada, "Jawas/Javas" or "Alashi" in Marathi and "Tisi" in Hindi, while in Telugu it is called "ousahalu". Flax was extensively cultivated in ancient Ethiopia and ancient Egypt. A prehistoric cave finding of dyed flax fibres in the Republic of Georgia have carbon dated the use of these plants up to 30,000 BC. [10]Furthermore, flaxseed is considered a super food with a wide array of benefits on human health. Still, there are more studies that are being undertaken to optimize the beneficial effect of this so called magic plant.[11]

Whole flaxseed (ground meal, powder or intact seed) contains 28% dietary fiber, (7– 10% soluble fiber, 11 - 18% insoluble fiber), 40% fat (73% of it being polyunsaturated fatty acids), and 21% protein. More than 50% of the fat in flaxseed is an essential fatty acid called omega-3 fatty acid (also known as alpha-linolenic acid, ALA); making flaxseed the richest plant source of omega-3 fatty acid. [12]Additionally, flaxseed also contains natural lignans; a type of polyphenol compound. Although other plants like pumpkin seeds, rye, soybeans, broccoli and some berries also contain plant lignans; flaxseed is considered the richest source of lignans with 100 times more than the next best source, wheat bran. One specific lignan compound, secoisolariciresinol diglycoside (SDG), is a precursor to a variety of phytoestrogens with antioxidant properties. It also contains small amounts of the lignans *matairesinol*, *pinoresinol* and *isolariciresinol.* [13]Prior cited study has reported that SDG isolated from Flaxseed has the ability to scavenge hydroxyl radical and hence has a high antioxidant potential.[14]

Additionally the omega-3 and lignan substance present in flaxseed are reported to demonstrate anti-cancer properties as cited in prior studies namely in animal model experiments. [15]Similarly a recent human as well as animal model study had reported that flaxseed compounds that maybe related to the shrinking of breast cancer tumours as well as treating prostate cancers. [16] Prior study cited postulates that it is the lignan antioxidants derived from flaxseed that contributes to this anti-cancer potency.[15] Additionally, the oil and phytoestrogens derived from flaxseed are being tested in relation to treating cardiovascular diseases. [17] In the field of food microbiology, a prior study in 2007 have reported the ability of flaxseed to act as a natural food preservative namely by inhibiting fungi such as *F. graminaerum*, *A. flavus*, *P. chyrsogenum* and the *Peniciillium sp.* in a potato dextrose agar and fresh noodle system. [18]Interestingly, the author of the former study promoted the use of flaxseed as a multifunctional ingredient in food with potential health benefit and natural fungistatic properties. Consequently in the past, Hurtado et al. have reported antifungal effect shown by flaxseed against *Aspergillus flavus* and *Aspergillus niger*although its antifungal mechanism was not expounded. [19] Hence it is clear that flaxseed extract is an untapped reservoir of potentially effective antifungal agent that could be used with minimal risk of side effect commonly seen in currently available conventional fungicide.

The aim of this study is isolate the flaxseed extract as oil and water based form which is rich in lignan as well as other phenolic compound and compares its antifungal activity against *Candida albicans* in vitro. This study will also compare the antifungal activities of the flaxseed oil extracts with the commercially available antifungal, Nystatin.

Materials and methods

This is a laboratory based in vitro comparative study. The flaxseeds used in this study were native to the region of Iraq, harvested during the 2010 to 2011 session. This cultivar was selected as it's considered to provide a good yield of oils and phenolic compound. The seeds were cleaned, packed and stored in paper bags in room temperature and transported to Kulliyyah of Science (KOS), International Islamic University Malaysia (IIUM) where it was processed.

Flaxseed extract preparation

Flaxseed extraction was done according to the previously reported method of extractions.[24-25] Seed were washed thoroughly with sterile distilled water and later were air dried at room temperature to prevent excessive dehydration and warping of phenol structure due to excessive heat. The samples were ground to mealy powder form by means of gentle cold pressed crushing method using pestle-mortar, again to avoid heat generation caused by power grinding. The sample powder will then undergo extraction as oil or water based preparation.

Oil extraction is initiated by first intimating the meal powdered seeds into a concoction of aliphatic alcohol solvent, namely a mixture of methanol with water. The slurry mixture is constantly stirred for 24 hours at temperature set between 40 to 60°C. The slurry is then filtered to separate the lignans rich alcohol solvent and its residual solids. The lignan-rich alcohol filtrate is then gradually concentrated by means of reducing the solvent. This is done by initially subjecting the filtrate to a base-catalyzed hydrolysis which enables the liberation of lignans in this solution to be available as a non-complexed form. The hydrolyzed concentrate later undergoes an ethyl acetate-water anion based partition that helps to further intensify the lignans within the filtrate. Thus a lignan-enriched solution is procured with revealed a purity of more than 90% lignans based on a chromatographic separation analysis.

As for the aqueous extract, defatted flaxseeds were required. The powdered defatted flaxseed was found to have the lignan, SGD in amounts up to 20 mg per gram of powder. The powdered seed sample was then homogenized in sterile water and later filtered by the same method stated above. The filtrate of aqueous lignan solvent is then concentrated by means under vacuum condition at 40° C with a rotary evaporator.

Specimen Culture Preparation

The agar and broth of Sabouraud Dextrose were prepared according to manufacturer instruction. The SDA broth is made by mixing 7.5g of Sabouraud Dextrose powder with 250 ml distilled water while the agar is made by mixing 15g Sabouraud Dextrose powder as well as 8.5g of agar with 500ml of distilled water. Both agar and broth were autoclaved at 121˚C for 15 minutes. At the same time, 7 petri dishes were prepared and placed under UV light in the fuming chamber for about 15 minutes to eliminate any presence of bacteria in them. Once autoclaving of SDA is done, 25ml of agar was poured into each of the petri dishes while carefully avoiding creation of bubble in the media. The solution is left to solidify in refrigerator for 24 hours.

A properly isolated, definitive strain of *Candida albicans sp.* was obtained from the laboratory of Kulliyyah of Science, International Islamic University Malaysia, Kuantan. The stock culture is being maintained on Sabouraud Dextrose medium at 4˚C. Next, the base culture inoculate was prepared using 3 sterile bottles that was individually filled with 10ml of sterile Sabouraud Dextrose broth. Then, 100µl of *Candida albicans* from the stock culture was transferred aseptically into each bottle using pipette. The petri dishes were sealed and left to incubate for 48 hours at 37 ˚C.

Antifungal activity testing

The antifungal activity was estimated via a disk agar diffusion test method. [20]Sterile filter paper discs, impregnated with 5µl, 10µl and 15µl of oil flaxseed or aqueous flaxseed extracts, were aseptically applied on the surface of the inoculated plates (Figure 1). The sterile discs are produced in triplicate for each type of extract and its amount. Similarly, the control discs containing Nystatin 100 units were used (Figure 2). Hence, three discs placed for each of the petri dishes were left to be incubated for 48 hours. Later, zone of inhibition was measured as diameter from the inner edge of the surrounding

pathogens, quantified as mm. Each assay in this experiment was repeated in quintuplicates.

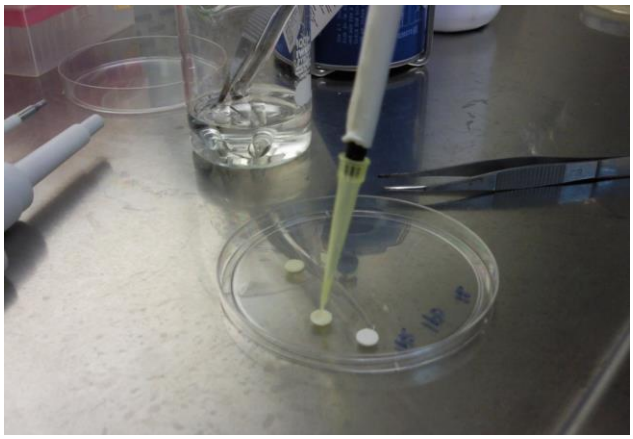

Figure 1. Impregnation of flax seed extract onto the filter paper disc via pipette.

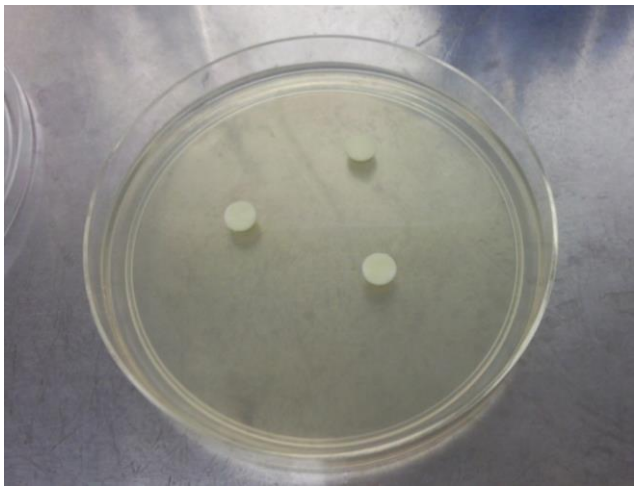

Figure 2. Discs containing extract set in triplicate on agar plate to be incubated.

Statistical analysis

All analyses were performed in 5 batches with triplicate sample per batch and the data regarding the zone of inhibition was recorded. Data analysis was carried out using IBM SPPS Statistics for Windows, Version 19.0. [21] via simple t-test to assess the microbial population growth of the different types of Flaxseed extracts at different volume in comparison to the Nystatin control group. The data are mainly presented as mean values with standard deviation.

Results

The antifungal properties of the extracts of *Linum usitatissimum* (Flaxseed) on the fungal cell culture has been observed and confirmed by the formation of growth inhibition zone on the petri dishes containing *Candida albicans* growth. Our results based on both Table 1 and Table 2 reveals that aqueous extracts of flaxseed possessed antifungal ability against *Candida albicans*. Table 1 describes the mean zone of inhibition or simply the antifungal activities exhibited by the flaxseed oil as well as aqueous extract in different volumes along with Nystatin. In general, it had been determined that the water based flaxseed extract showed a higher positive antifungal effect that the oil based extract and even the Nystatin 100 units. The analysis of variance test done to compare the mean value between the flaxseed aqueous extract and Nystatin has revealed significant difference in *Candida albicans* growth inhibition diameter between the extract volumes as indicated by the p-value being less than 0.05. The highest mean diameter of growth inhibition zone was formed by the 15 µl flaxseed aqueous extract imbedded discs with a mean diameter of 15.60 ± 0.55 mm. Nystatin 100 units result was used as control test revealing a mean inhibition zone of 8.70 ± 0.45 mm. Interestingly enough, there was no growth inhibition zone recorded in all the petri dishes containing the flaxseed oil extracts in any of the volumes used (Figure 3).

Types of Substance	Extract Form	Volume/ Unit	Mean zone of inhibition (mm)± Standard Deviation	P value
Flax seed	Oil extract	5µl	NGI	
		10µl	NGI	-
		15µl	NGI	
	Aqueous extract	5µl	10.20 ± 0.45	< 0.05
		10µl	13.40 ± 0.22	
		15µl	15.60 ± 0.55	
Nystatin (Control)		100 unit	8.70 ± 0.45	

NGI: - No Growth Inhibition

Table 1. Antifungal activities of different volume of aqueous and oil flaxseed extract as well as Nystatin against Candida albicans.

Volume amount	Growth inhibition diameter		P-value (*one-tailed*)
	Flax seed aqueous extract	Nystatin	
5 µl	10.2 ± 0.20	8.7 ± 0.20	0.01
10 µl	13.4 ± 0.05		< 0.01
15 µl	15.6 ± 0.30		< 0.01

Table 2. T-test comparison of antifungal activities at different volume of flaxseed aqueous extract in comparison to Nystatin.

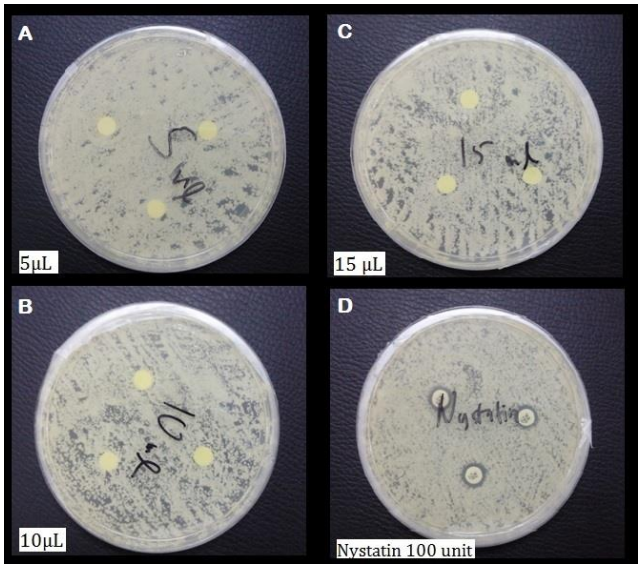

Figure 3. Candida albicans culture petri dishes with discs impregnated with flax seed oil extracts 5, 10 and 15 µl (A,B,C) along with Nystatin (D) after 48 hours of incubation. No growth was seen around the oil extract discs as growth inhibition is indicated by clear area around the discs.

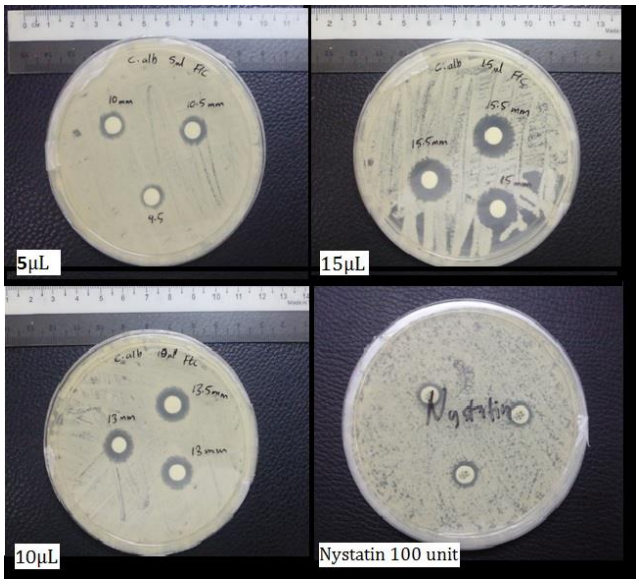

Figure 4. Candida albicans culture petri dishes containing discs impregnated with flax seed aqueous extracts of 5, 10 and 15 µl (A,B,C) along with Nystatin (D) after 48 hours of incubation. Fungal growth inhibition is indicated by the clear area around the discs.

Table 2 shows the t-test analysis of each of the flaxseed aqueous extract volumes in comparison to Nystatin. Again the growth inhibition of *Candida albicans* is significantly different at different volume of aqueous extract in comparison to Nystatin, as depicted by the p-value being less than 0.05. It is clearly seen that at all different volumes the growth inhibition of fungal growth was considerably higher than the growth inhibition exhibited by Nystatin. Additionally, the growth inhibition of *Candida albicans* was progressively increasing in diameter from 5 µl to 15µl. This result can be clearly seen in the images of the sample results obtained (Figure 4).

Discussion

The results shown in this study (Table 1, Table 2) clearly indicates the aqueous flaxseed extracts provided clearly favourable antifungal effect against tested *Candida albicans* culture. Interestingly, the antifungal effects of these aqueous extracts were greater than the commercially used Nystatin antifungal drug. On the other, a pure oil flaxseed extract did not exhibit any antifungal effect on *Candida albicans*. This result appears to mimic the finding of a prior study that observed that essential oil obtained from *Stevia rebaudiana Bertoni* leaves had the lowest antimicrobial and antifungal effect as compared its aqueous extract. [22]This finding may be due to a number of possible reasons. Prior studies cited have maintained that when it comes to plant extracts in general, it is the water extract that exhibited the highest phenolic content along with the highest antioxidant activity. [23] It is noted that polyphenols are a plant compound that is related to antioxidant properties namely due to this compounds increased affinity to bind and neutralize free radicals. [24]Since this compound is found abundantly in flaxseed, [25]one could surmise that the antifungal effect seen in this study could be attributed to the presence of this component.

Additionally, another biopolymer substance found abundantly in flaxseed is called lignin which mainly functions in providing rigidity to cell wall while acting as a buffer to mechanical stress a plant may experience. Surprisingly, another study in 2011 have reported that lignin in aqueous phase is depolymerized to form a monomeric aromatic compound that in turn could

be reformed to produce hydrogen as well as other simple aromatic chemicals. [26]Fang et al. in 2007 reported that a homogenous mixture of lignin, water and phenol can lead to breakdown of lignin to phenolic derivatives as well both via hydrolysis and pyrolysis.[27]

The same study noted that without the presence of phenol, lignin remains in a heterogeneous phase in the water in the set condition. Phenolic compound are known to be toxic against microbes by causing enzyme inhibition, cell wall disruption and protein deconstruction. [28]Additionally, other studies concurs that apart from phenolic derivatives, lignans such as SDG (also present in Flaxseed) are involved in antimicrobial activity.[29,30]

Conclusions

In conclusion, this study has proven that flaxseed constituent has a significant antifungal effect namely against *Candida albicans* growth. Additionally, a water homogenized flaxseed extract showed a significantly greater antifungal property compared to the routinely available Nystatin. Strangely enough, the highly concentrated essential flaxseed oil extract was not able to exhibit antifungal effect at all. Hydrolyzed flaxseed aqueous extract is also able to increase the antioxidant activity. It is hoped that further studies will be done in the future to isolate the responsible antifungal derivatives within flaxseed extract.

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