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Superior Efficacy of Egyptian *Anticandidal* Drugs Via combined therapy with *Syzygium aromaticum* Extract

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ABSTRACT

Candida spp. cause a very serious health problematic issue called candidiasis which affects many patients especially females. Egyptian pharmaceutical market EPM is full of many antifungal drugs with variable degrees of efficacy for many reasons. Thereafter, this ongoing study aimed to overcome the resistant problem emerged by Candida spp. by combination with clove extract. Isolation of *Candida spp*. from different areas overall Egypt including females, males and children (1- 92 years-old), A huge number of yeast isolates were recovered; hence, CHROM agar medium CAMC was used for *Candida* differentiation as well as preliminary identification. Susceptibility test was performed using both of antifungal discs and antifungal drugs which had been already established in Egyptian pharmaceutical market in addition to some plant extracts using different solvents. Combination between plant extract and antifungal drugs was evaluated, cytotoxicity By MTT assay and finally prediction of subcomponents by GC-MS. The activity of each drug was determined, and the most resistant isolates were reported, chloroform-methanol 2:1 (v/v) and clove were succeeded together to exhibit antifungal activity. Mixture of clove extract and antifungal drugs was evaluated and fortunately, the In vitro results were hopeful due to the superior activities of this mixture which surpassed both clove extract and the drugs individually. The cytotoxic test exhibited perceptible cytotoxicity that is matching the topical usage of antifungal drugs which were applied superficially not to be administrated orally or via injection, and finally, GC-MS displayed presence of sitosterol which reflects the antifungal property.

INTRODUCTION

Infections caused by fungi are termed mycoses and there are two types of mycoses; primary and secondary [1]. Primary mycosis or fungal infection affects both immune competent and immune compromised human hosts. Secondary fungal infections include the opportunistic fungal infections caused by other fungi species, which include *Candida*, Aspergillus, Fusarium and Zygomycetes species in the immune compromised and highly debilitated hosts [2]. The class Eumycota includes genera such as *Saccharomyces, Pichia*, *Endomycopsis, Nematospora*, and Candida. About 60 − 70% of patients admitted into Intensive Care Units (ICUs) have symptoms caused by *Candida* species [3]. The single most common genus to cause opportunistic fungal infection among immune compromised hosts worldwide however is *Candida*, although the list of opportunistic fungi causing life-threatening infections among the immune compromised increases periodically [4].

Candida species member of the normal flora of an individual from the gastrointestinal tract, vagina, oral cavity, skin and mucosal surfaces of human [5]. *Candida spp*. are microbiota in healthy humans during in immune compromised situations can cause human infection, the genus *Candida* includes more than 100 different species, however, only a few of them can infection

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human [6]. The last decade has seen *Candida spp*. are continuously medical importance, they could be opportunistic can causes life to threaten systemic infections and chronic mucocutaneous infection in immune compromised patients [7]. *Candida species* are present human fungal pathogens can cause genitourinary candidiasis which involves vulvovaginal candidiasis in female and balanoposthitis and balanitis in male, oral candidiasis, and the digestive tract and candiduria in both genders [8]. Several brands of CAMC are available for rapid identification of yeast [9]. These special media yield microbial colonies with varying pigmentation secondary substrates that react with enzymes secreted by microorganisms. Development of resistance through natural selection is a known evolutionary process which depends on genetic variability. Exposure to antimicrobial agents typically promotes the development of drug resistance in all pathogenic microorganisms, including fungi. Some of the factors include patient compliance, immune status, host adherence to the antifungal agents and drug-drug interactions; whereas factors inherent to the organism include pharmacokinetics and biofilm formation that partly contribute to resistance [10]. Changes in sterol biosynthesis may occur as a result of abrasions in the 5,6 desaturase and may lead to accumulation of 14 α-methyl fecosterol instead of ergosterol [11-12] The plants-antimicrobial combination can modify the antimicrobial activity which potentially exhibiting antagonism, synergism, indifferent or additive. The synergistic interaction provides increased antimicrobial activity using lower concentration when used together, as well as additive interaction does not provide increased nor decreased antimicrobial activity if used together, and antagonist interaction provides decreased antimicrobial activity [13-14]. Multidrug therapy is considered advantageous because interactions between the substances accelerate the protective and repair mechanisms [15], expands the antimicrobial spectrum, prevents the emergence of resistant mutants, etc. [16]. The current research targeted improvement of antifungal drugs in Egyptian pharmaceutical market against candidiasis as well as manipulation of resistance problem emerged by *Candida spp*. this improvement will be achieved through the combination between antifungal drugs and clove extract.

MATERIALS AND METHODS

Different medical specimens were collected from different localities private medical laboratories as well as some hospitals in Egypt (Cairo – Benisuif – Tanta - Elmenia). Fungal samples were collected from different infected sites of human body such as urine, tongue, blood, sputum, pus, nails, vaginal, GCF, bronchial swab, stool, nasal and pleural fluid. A total of 142 specimens were taken from 129 patients their ages ranged from 1 to 92 years from October 2017 to December 2017. A total of 117 Candida isolates were collected to be investigated.

MEDIA

Sabouraud Agar Modified

Gentamicin: 5.0 mg is an aminoglycoside antibiotic that inhibits the growth of gram-negative bacteria. Chloramphenicol; 50.0 mg is inhibitory to a wide range of gram-negative and gram-positive bacteria, and cycloheximide 20 mg is an antifungal agent that is primarily active against saprophytic fungi and does not inhibit yeasts or dermatophytes [17].

CHROM agar medium for *Candida* CAMC

CAMC was used as a differential medium for differentiation of *Candida albicans, C. tropicalis and C. krusei* [18]. It was prepared according to the manufacturer's instructions (CHROM agar *Candida* Company, Paris, France), it contains (g/L): peptone 10, glucose 20, agar 15, chloramphenicol 0.5, chromogenic mix 0.4, distilled water up to 1.0 L.

Purification and identification of yeast isolates

Growth conditions, macroscopic examination, microscopic examination, CAMC, germ tube formation, SEM and some biochemical characteristics (by Vitek-MS) were used for identification of yeast isolates.

Morphological characteristics

All yeast isolates were cultivated on primary identification medium to investigate both cultural and microscopic features according to [19].

Macroscopic examination

Isolates were cultured on malt extract special medium for primary identification, some criteria of yeast isolates were investigated such as colonies colors, textures which were described by direct examination [20].

Germ tube formation

The germ tube test is one of the most rapid and a simple test for presumptive identification of *C. albicans*. One ml of sterile serum was inoculated using a sterilized blue tip, then incubate at 37 °C for no longer than 3-4 h, then one drop of the yeast-serum mixture was placed on a slide to be investigated under light microscope [21].

Biochemical and physiological characters (VITEK® 2 YST)

Ten different yeast isolates were selected preciously to be identified by Vitek® 2 YST equipment (Biomerieux USA) in Ain

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Shams University Hospital. Directly deposit the yeast isolate on the target slide, adding the ready-to-use matrix solution and inserting the slide into the Vitek® 2 YST system. Identification results are displayed within minutes depending upon the linked library include huge database which contains many specific microorganisms related to multiple regions countries, sources, clinical and environments. In addition, proprietary algorithm vastly increases the accuracy of identification.

Scanning electron microscope SEM

Fixation and dehydration procedures were performed using the programmable Electron Microscopy. Only six identified isolates completely by Vitek® MS were subsequently investigated by SEM in RCMB to display more details of microscopic features of yeast isolates, SEM technique was performed in different steps including fixation by immersion in osmium tetraoxide (Os04) at 4°C for 12 hours in the dark, dehydration by tissue processor (Sciences tissue processor model Lynx) through immersion in gradual concentrations of ethyl alcohol followed by gradual concentrations of acetone, critical point dryer (EMS 850 apparatus) in which acetone was replaced by carbon dioxide, mounting on stubs to be coated by gold for good conductivity by diode gold sputter coater (SPI Module™ Sputter Coater) and finally examined by high-vacuum mode of a JEOL JSM-5500LV Scanning Electron Microscope [22].

Susceptibility of yeast isolates to antifungal discs

Eight antifungal discs (Bioanalyser®) include different formula; AMB: Amphotricin (100 U), MCZ: Miconazole (50 µg), VOR: Voriconazole (1 µg), NY: Nystatin (100 U), KTC: Ketoconazole (10 µg), ECO: Econazole (10 µg), FlU: Fluconazole (25 µg), CLT: Clotrimazole (50 µg) were tested against 24 yeast isolates to evaluate the response of these yeast isolates to the antifungal discs using agar disc diffusion.

Susceptibility of yeast isolates to antifungal drugs in EPM

Eight different antifungal drugs were purchased from different pharmacies; these drugs include different formulas (active ingredients) and forms (tablets, cream, spray, drops, ointment, gel. etc.). Gyno-Trosy tablet, Pevaryl Cream, Tineacure cream, Ultragyrisfolain suspension, Mycostatin suspension, Terbin spray, Micoban spray and Candistan solution are the antifungal drugs used during this experiment. This step was performed using agar well diffusion method [23].

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Antifungal activity of plant extracts

Plants / plants' extracts preparation: Five different plants (Table 1) were selected to be investigated as antifungal agents, their extracts were prepared using three different solvents; water, diethyl ether and chloroform-methanol 2:1 (CM). 25 g were agitated with solvents individually by different ways such as grinding, shaking, soaking over night to give an opportunity for effective extraction. All extracts yielded were filtered to exclude any cell debris to get the clear filtrate and the filtrate was then kept at room temperature to allow to be dried under partially aseptic conditions. Thereafter, the dried residues of filtrates will be ready for further investigations.

Table 1: Full data about the selected medicinal plants.

Common Name	Scientific Name	Part involved
Garden thyme	*Thymus vulgaris*	Arial part
Garden cress	*Lipidium sativum*	Red seeds
Fennel flower	*Nigella sativa*	Black seeds
Clove	*Syzygium armaticum*	Flower buds
Ginger	*Zingiber officinale*	Rhizomes

Evaluation of antifungal activity of plants extracts: The residues of dried filtrates were weighed and then dissolved in methanol to prepare different concentrations; 10, 40, 100 mg/ml. Only the first concentration (10 mg/ml) was primarily tested as antifungal agent against a small batch of yeast isolate to give an impression about the quality of solvent applied and the plant activity, so only five yeast isolates were selected to perform this experiment. All three prepared concentrations 10, 40 and 100 of clove extract via CM were then investigated against all 24 selected yeast isolates to detect the most potential concentration which exceed the values obtained by the action of drugs and equal or exceed the values obtained by antifungal discs.

Combination between drugs and clove extract (CM): This trial was done by mixing clove extract CM 100 mg/ml and the targeted drugs in a ratio of 1:1, the final concentration for both is the half of the original prior mixing.

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In vitro estimation of cytotoxicity of the crude clove extract: Determination of cytotoxicity of clove extract against the normal cell line (Vero) according to MTT protocol as follow; inoculation of 1 x 10^5 cells/ml (100 µl/well) for each well in the 96 well tissue culture plate and incubated at 37 °C for 24 h, washing twice, two-fold dilutions of tested sample was made in maintenance medium (RPMI) medium with 2% serum, added to the wells in corresponding to negative control, incubation at 37 °C and examined. MTT solution was prepared (5 mg/ml in PBS) (BIO BASIC CANADA INC), 20 µl of MTT solution were added, shaking at 150 rpm for 5 min, incubate (37 °C, 5% CO2) for 1-5 h, the media was discarded, re-suspend formazan in 200 µl DMSO, shaking at 150 rpm for 5 min, read optical density at 560 nm [24].

GC-MS analysis for expectation the composition of clove crude extract: The sample was injected by injector (250 °C) into GC-MS equipment (Thermo Scientific TRACE 1310 Gas Chromatograph) which was attached with ISQ LT single quadrupole mass spectrometer (Thermo fisher scientific). The run was done by some help of helium gas with a flow rate of 1.5Psi/minutes. Analysis was done by GC-MS provided with a column (DB5-MS, 30m: 0.25mm ID (J&W Scientific), with EI ionization model and ionization voltage of 70e. v. The temperature program was; 50°C (1min), 150°C (1minute) at 7°C/min, 250°C (5minute) at 5°C/minute, 290°C (2minutes) at 10°C/minute. The chromatogram was presented by Xcalibur software which was connected with built-in library (WILEY & NIST MASS SPECTRAL DATA), referring to detector involved with a temperature of 300°C [25].

RESULTS AND DISCUSSION

Sampling

A total of 142 specimens were collected from patients (males, females and child) their ages ranged from 1 to 92 years. In the present study; Figure 1 represented clinical fungal specimens, Figure (1) exhibits that urine harvested the top level of infections by *candida spp*. followed by sputum, pus, vaginal swab, stool, nails. Table 2 displayed that women were more exposed for *candidal* infections (62.6%) followed by men (28.7%), while the percentage of kids who were attacked by yeast infections is the least value (8.7%).

Table 2: Preliminary identification of Candida species by ChromAgar media.

Preliminary identification	Colony characteristics on ChromAgar	Number of isolates	Male	Female	Child
C. albicans	Apple green colonies; consistent	65	23	37	5
C. glabrata or	White large glossy pale pink to violet colonies	47	9	33	5
C.parapsilosis					
C. tropicalis	Dull blue, to purple color with pale pink edges	2	1	1	
C. krusei	Large, flat, spreading, pale pink colonies with matt surfaces	3	1	2	

Preliminary identification of yeast isolates

CHROM agar medium for Candida: CAMC contains enzymatic substrates that are linked to chromogenic compounds. The action of different enzymes produced by yeast species results in color variation which is useful for the presumptive identification of some types of yeast species. In the present study CAMC was used for detection of five species which could be identified as follow: *Candida albicans* (green), *Candida glabrata* (white purple), *Candida parapsilosis* (white purple), *Candida tropicalis* (blue) and *Candida krusei* (pink). Color of colonies on CAMC was similar as given by the manufacturer (Table 2). It is necessary to consider that is probable for presence of other species than the five of CAMC spectrum.

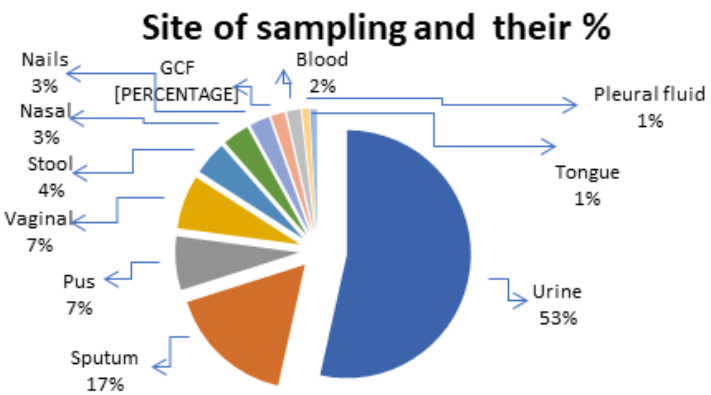

Figure 1: Pie chart represents the percentage of Candida spp. according to the site of sampling overall the body infected parts..

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117 *Candida* isolates were recovered from 115 patients, that means in general only one isolate is responsible for the etiology of infection and rarely participation of more one species to cause the infection. CAMC was considered as a helpful tool for categorization of a huge number of *Candida* isolates into only four categories according to the resulted colors with five probabilities. The prevalence of Candida in critically ill patients in the study was calculated in a percentage as follow: *C. albicans* (55.6%) was the highest occurring pathogens isolated, followed by *C. glabrata*, *C. parapsilosis* (40.1), *C. krusei* (2.6%) and *C. tropicalis* (1.7%). (Table 2). This results is in line with (Pahwa, Kumar, Nirkhiwale, & Bandi, 2014) who reported that the majority of candiduria were caused by *C. albicans* (24.8%), non-albicans species, especially *C. glabrata* or *C. parapsilosis* (27.3%) was emerging as a nosocomial infection. Several other investigator form India also reported a high prevalence of *Candida causing* UTI. Also, *Candida albicans* is by far the most common cause of *candidal* infection and has been recovered from up to 70 − 90% of *candidal* infected hosts [26].

Macroscopic and microscopic examination by both light microscope and SEM

According to the results of CAMC, 24 isolates were preciously selected to express all 117 isolates. Those 24 reflects all types of *Candida spp*. That had been detected on CAMC, also most of risk factors such like "sampling sites, gender, age" were taken into consideration. Light microscope was used to validate the first tool (CAMC). Unfortunately, light microscope displayed more details for the results within the same group which means arising of new species rather than the species belonged to CAMC so, these new results were taken in consideration besides the default five species for further and deeper identification by germ tube formation, SEM and some biochemical characteristics by Vitek-MS technology (Figure 2). *Candida albicans*; colonies are white to cream-colored, smooth, glabrous yeast-like, spherical subspherical budding, 2-7 × 3-8 µm in size and these isolates were confirmed by germ tube formation test. *Candida parapsilosis*; colonies are white to cream-colored, shiny and smooth, glabrous yeast-like, predominantly small, globose to ovoid budding, 2.0-3.5 × 3.0-4.5 µm, with some larger elongated forms present. *Candida glabrata*; colonies are white to cream-colored smooth, glabrous yeast-like, ovoid to ellipsoidal budding, 3.4 × 2.0 µm in size. *Candida krusie*; colonies are white to cream-colored rough, glabrous yeast-like, predominantly small, elongated to Ovoid budding, 2.0-5.5 × 4.0-15.0 µm in size. *Candida tropicalis*; colonies are white to cream-colored smooth, glabrous yeast-like, spherical to subspherical budding yeast-like cells, 3-5.5 × 4-9 µm in size. *Candida famata*; colonies are white to cream-colored smooth, glabrous yeast-like, ovoid to broadly ellipsoidal budding, 3.5-5 × 2-3.5 µm in size.

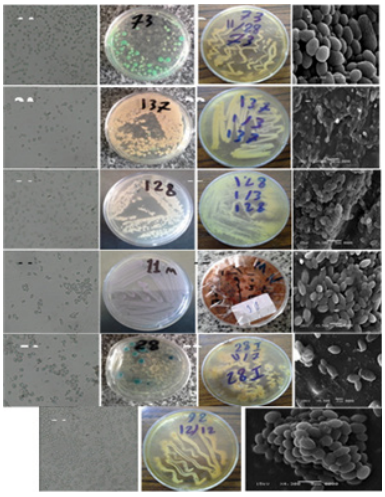

Figure 2: Culture and microscopic characteristics of 1- *C. albicans*, 2- *C. parapsilosis*, 3- *C. glabrata*, 4- *C. glabrata*, 5- *C. krusie*, and 6- *C. tropicalis*, where, A, Cell shape under light microscope (magnification: 400x), B, The color on CHROM agar, C, Colonies color and texture on Malt Extract Powder agar and D, shape and size of yeast cells under SEM.

Some yeast species were selected and identified by SEM and VITEK-MS as a relatively accurate tool rather than the Chromogenic property that used for discrimination purposes only. *Candida albicans, C. tropicalis, C. krusei, C. glabrata, C. parapsilosis and C. famata, C. famata* was identified by CAMC as other species but totally chromogenic property succeeded in high percentage in 4 species out of 5 (80%). Vitek-MS was used to deeply identify the selected yeast isolates, it was confirmed the previous morphological identification.

Susceptibility of *Candida* isolates to antifungal discs

The preciously selected 24 *Candida spp*. were considered as test organisms for 8 different antifungal discs. This test was performed to evaluate the susceptibility of *Candida* isolates toward the active ingredients in a form of discs to categorize the active antifungal into potential, mild or moderate and weak depending upon the diameters of clear zones resulted.

Amphotricin was the most resisted antifungal and it was not recommended for use, while clotrimazole, miconazole, nystatin and econazole may be described as moderate antifungal agents. On the other side voriconazole, ketokenazole and fluconazole

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may be considered as potential antifungal agents with clear superiority for voriconazole followed by fluconazole. It was clearly observed that isolate (*C. parapsilosis*) is the most resistant isolate but it exhibited mild sensitivity to ketokenazole and clotrimazole. These results were supported by [27] who reported that non-albicans *Candida* were showing the high degree of resistance against Amphotericin B. The most active antifungal drugs for all the *Candida* species were Nystatin. We also found that Clotrimazole was more active against non-albicans species (80%). The level of fluconazole resistance among the isolates tested was relatively low, like that. Only a few isolates of *C. albicans* (two), *C. parapsilosis* (two), *C. tropicalis* (one) and *C. glabrata* (one) were resistant to fluconazole [28-30]. Therefore, fluconazole can still be used as a first-line therapy for infections caused by *Candida species* other than *C. glabrata* and *C. krusei*. The percentage susceptibility of *C. glabrata* isolates to fluconazole [30].

Susceptibility of *Candida* isolates to antifungal drugs in EPM

Candida isolates were tested by 8 variable antifungal drugs (Gyno-Trosyd, Pevaryl Cream, Tineacure, Ultragyrisfolain, Mycostatin, Terbin, Micoban, and Candistan) via agar well diffusion method which showed variable responses ranged from resisted drugs to potential ones. Antifungal susceptibility showed a high resistant rate to Tineacure and Ultragyrisfolain (all tested isolates are resistant), followed by Pevaryl Cream (only 2 isolates are resistant and the clear zone ranged from 11 to 17 mm) and Mycostatin (only one isolate is resistant and the clear zones ranged from 10 to 15 mm) which may be classified as weak antifungal drugs. While, Gyno-Trosyd might be classified as mild to moderate antifungal drug; only one tested isolate gave a clear zone 5 mm while the range of clear zones among the other tested isolates is 12 to 21 mm. On the other hand, Terbin, Micoban, and Candistan were classified as effective antifungal drugs; all these types of drugs belong to the form of spray; Terpin spray gave inhibition zones ranged from 15 to 30 mm, Micban gave inhibition zones ranged from 13 to 25 mm and finally Candistan from 12 to 28 mm. All the tabulated data had been turned to a form of histogram to clarify the variability in responses of the tested *Candida spp*. toward antifungal drugs.

Antifungal activity of extracts of some medicinal plants

The uses of plant-derived products as disease control agents have been studied, since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance [31]. In the current study; five types of plants (Clove, Nigella sativa, Thymus, Zingiber and Garden cress) were extracted via 3 different solvents; water, diethyl ether and chloroform-methanol (2:1) [CM]. Water and ether extracts were estimated as antifungal against a random sample of tested *Candida sp*. (only 5 isolates) but unfortunately, both of them had no antifungal property. On the other hand, extract of clove chloroform-methanol (2:1) had succeeded as antifungal against the selected random sample hence; this extract was investigated against all tested Candida sp. (24 isolates) among all types of plants selected. Four types of plant extracts (thymus, zingiber, Nigella sativa and garden cress) were investigated as antifungal, only the crude clove extract (CM) gave moderate results expressed as inhibition zone ranged from (13 to 21 mm) which is less than the range of inhibition zones resulted from the positive control; Voriconazole (1 µg), accordingly, this result encourage using of the higher concentrations (40 and 100 mg/ml) of the crude clove extract (CM). Antifungal activity exhibited by Syzygium aromaticum may attribute to the presence of secondary metabolites. These compounds can interfere with pathogens by different mode of action. Freiesleben and äger, had previously investigated the correlation between the biosynthetic group of secondary metabolites in plants and their antifungal mechanisms of action. From looking at the composition of the fungal cell, at least 6 different antifungal mechanisms can be suggested [32].

Two other concentrations of the crude clove extract (CM); 40 and 100 mg/ml were used for revealing the inhibitory effect of different concentrations of plant extract against each *Candida species*. Table 3 displays the effect of all investigated concentrations of clove extract against test *Candida species* which were affected by different degrees. It is clearly that the antifungal powerful of the crude clove extract is in direct proportion with its concentration this means when the concentration increased the inhibition zones will be enlarged. For the first look to table 3 it is obviously observed increasing of inhibition zone diameters in concentration 100 rather than 40 or even 10.

Table 3: Effect of different concentrations of clove extract against test *Candida species* expressed as diameters of inhibition zone in (mm).

Diameters of inhibition zone (mm)			Tested yeast isolate
100 mg/ml	40 mg/ml	10 mg/ml	
29	26	21	MWOSP3
18	17	15	FWOSP8
25	23	15	MPOSP11a
20	17	14	FPOSP14
27	23	16	FBOUR28
28	25	20	MGCHCS40
26	23	17	FGOBR41
24	22	19	MGOST44
22	19	12	FWYVA45

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30	26	15	FWYNA50
20	17	11	FGOST64
25	20	14	FGOSP73
19	15	12	MGOSP75
25	24	15	FWCHBL92
29	25	20	FGYUR103
22	18	13	MBONA109
18	16	13	FGOUR116
23	19	13	FGOPU121
22	24	19	MWOUR128
19	17	15	FWYUR135
30	25	15	MWCLPL137
27	25	19	FGYVA138
24	19	13	MGOPU139
23	18	12	FGOTO142

Testing the efficiency of antifungal drugs in mixture with the crude clove extract

The combination process which reflects success of this process in improvement of antifungal property for antifungal drug insofar some cases of resisted antifungal turned to moderate effect and this is a very good result. Fortunately, in some trials; combination process succeeded in improvement of both components together rather than individual form; that means exhibition of synergistic effect between two components. The rest of trials appeared to be good in a side of the drug (some of the turn from resisted to moderate effect) but not good in the other side of the crude extract (inhibition zones had the same value or less), those trials showed a significant improvement for the drug accompanied with decreasing of the effect of the crude extract.

Finally, this experiment relatively succeeded in a level of drug that clearly improved in most cases so, further studies should be done in a pure antifungal compound included in the crude extract this pure form could be gotten by purification techniques like fractionation or chromatography to obtain higher activity with less cytotoxicity. The crucial need for antimicrobial agents increased day by day because of many reasons, the most important reason is evolving of microbial resistance, and as mentioned in Suleiman [33] who stated that the antimicrobial agents which belong to the natural origin could overcome the resistance problem. Also, Ruíz-Cendoya et al. [34] established efficacy of voriconazole, amphotericin B which gave not impressive results but the efficacy of combined action surpass the monotherapy.

***In vitro* estimation of cytotoxicity of the crude clove extract**

The toxic effect crude clove extract was evaluated by MTT assay against Vero cell line. Figure 3 discussed the effect of different concentrations of Crude clove extract to calculate CC50 which was 0.269 mg/ml. Unfortunately, the crude clove extract showed high toxic effect at 50 mg/ml reached to 87% so, the research advised to comply with the concentration not more than CC50 value to study the action of combination. Also, this experiment may advise to purify the crude extract to get the pure form of active ingredient that had the antifungal property, chromatographic techniques may be helpful tools for purification purposes, as well as different solvents rather than CM, water and ether could be investigated, extraction solvent might contribute in cytotoxicity in a way or another.

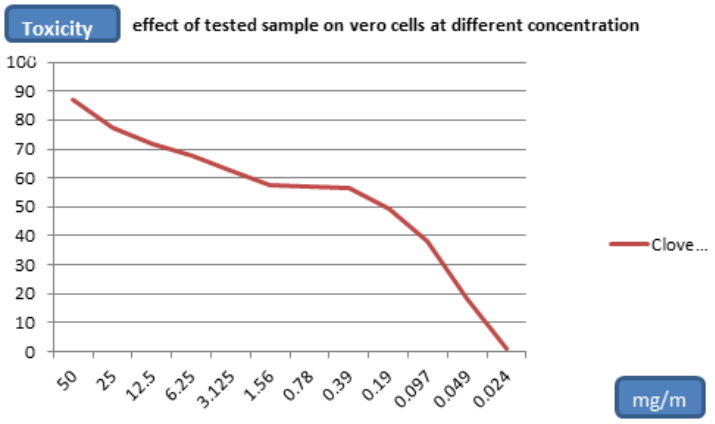

Figure 3: Line chart shows effect of tested sample on Vero cells at different concentration.

Additionally, these types of extracts that have cytotoxic effects may be oriented in application for topical use only such as cream, ointment, spray, etc., fortunately, most of fungal diseases especially that belonged to candidiasis are not systemic, they mostly infect skin, hair, soft tissues, ear and in some cases tongue and vagina which all could be classified as topical infections.

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Reports of essential oil cytotoxicity are in their infancy and very few attempts have been made to determine active cytotoxic components of essential oils. In this study, the crude clove extract showed high toxic effect at 50 mg/ml reached to 87% so, the research advised to comply with the concentration not more than CC50 value to study the action of combination. Further studies are, however, required to determine whether this component of clove oil makes any contribution towards the activity of the whole oil. All experiments performed in this study utilized a 1 h incubation following preliminary experiments using a range of times, which indicated no notable further increases in cell death with prolonged incubation, this timing agrees with previous work [35].

Prediction of subcomponents of the clove extract by GC-MS

GC-MS is successful tool to analyze the plant extracts to speculate the constituents [33], figure 4 and table 4 show subcomponents accompanied with their retention time RT, molecular formula and molecular weight. Only five components were detected; eugenol, caryophyllene oxide, N-oxyclozapine and sitosterol with molecular weight ranged from 164 to 414. Investigation chemical nature of active antifungal component in S. aromaticum was carried out by using GC-MS to predict the chemical profile of the crude ethanolic extract of clove.

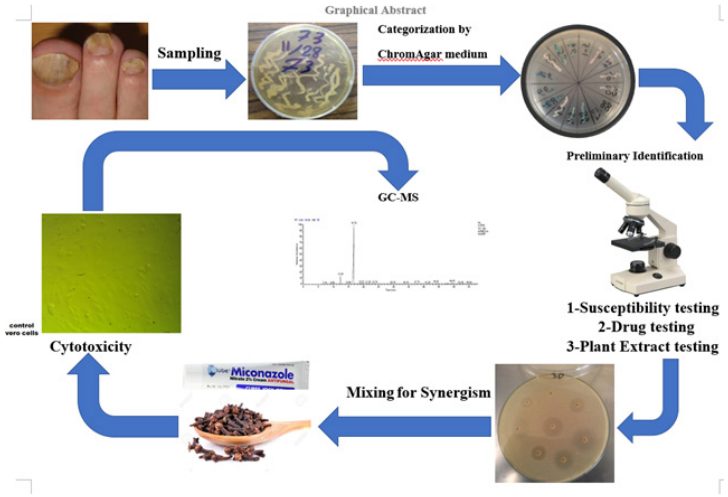

Figure 4: GC-MS chromatograph of clove extract to expect their ingredients.

The results revealed that the crude extract contained 30 different chemical compounds with different concentrations such like eugenol, trans-caryophyllene, humulene, anthracenedione, cedran-diol, citroflex A and lucenin 2. This result is consistent with that of [25] who found that the major compounds in clove alcoholic extract analysis by GC-MS were eugenol, acetyl eugenol, caryophyllene, and humulene followed by a-farnesene and caryophylleneoxide. These notable differences in composition may be due to extraction methods as well as genetic diversity and agronomic treatments [36].

Table 4: GC-MS analysis of clove extract subcomponents.

RT	Prediction of compounds	M. formula	M. wt.
12.38	Eugenol	C10H12O2	164
16.79	Phenol,2-methoxy-4-(2-propenyl-acetate	C12H14O3	206
19.33	Caryophyllene oxide	C15H24O	220
44.08	N-Oxyclozapine	C18H19ClN4O	342
49.45	Sitosterol	C29H50O	414

This current study conclude that clove extract has very important value as antifungal agent especially candidiasis whether it was used individually or in combination, also it was noticed that solvents had an aggressive impact to extract the bioactive compounds where, some may fail and the other may succeed according to the polarity degree which affects the extractability quality/efficacy over all levels whether numbers of compounds recovered or their concentrations and this extrapolation clarify that some examined medicinal plants failed to exhibit any anticandidal activity. Regarding to cytotoxicity test, it is very important test to be carried out *in vitro* to test the applicability feasibility in a safe way, although this test limits the spectrum of application but it doesn't completely inhibit the use of drug because the drug may be applied externally in a form of gel, cream, lotion, ointment, etc. Combined therapy is very trusted because it gave better results with low dose so, it is lowering the side effect of the synthetic drug and increasing its efficacy. Thereafter, the current research recommends the use of combined therapy especially in the treatment of infectious diseases or the physicians may recommend the use of clove extract as a syrup besides the antifungal treatment course.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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