Comparative evaluation of the effect of Nigella sativa extracts and nystatin as a traditional drug on Candida albicans in the primary school students in Mosul and Tikrit cities.

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ABSTRACT

Objectives: To detect and compare the inhibitory effect of Nigella sativa extracts and compare their effects with traditional drugs on Candida albicans.

Introduction: The developing microbial resistance to the existing anti-microbial agents has become a real challenge and a serious problem. Seeds of *Nigella sativa* have been used for a long time in folk medicine for the treatment of such infections. Production of new potent agents is urgently needed, especially for hospitals and health centers. Therefore, the antifungal effect of aqueous and alcoholic extracts of the seeds against *Candida albicans* from primary school students were investigated.

Materials and Methods: The in vitro antifungal effect of the extracts at a concentration of (10, 15, 20, 25, 30, 35, 40)mg/ml on *C. albicans* isolated was assessed and compared with traditional drug, nystatin using agar diffusion assay.

Results: The aqueous extract did not show any inhibitory effect against the isolated *Candida*. The alcoholic extract indicated significant inhibitory effect. nystatin show inhibitory effect higher than alcoholic extract at a concentration 40 mg/ml.

Discussion: The results of this study revealed clear potentiality of *N. sativa* as a source for antifungal drugs and support its use in folk medicine for the treatment of fungal intestinal infections. This finding warrants necessity of further investigation of this product of folk medicine.

Keywords: *Nigella sativa* extracts, nystatin, *Candida albicans*.

الخلاصة

اهداف البحث: صممت الدراسة للكشف عن التأثير التثبيطي لمستخلصات الحبة السوداء ومقارنتها مع الادوية الشائعة الاستعمال ضد الفطر *Candida albicans*.

المقدمة: ان التطور الحاصل في مقاومة الاحياء المجهرية ولما موجود من مضادات حيوية اصبح مشكلة خطيرة، حيث تستعمل بذور الحبة السوداء في الطب الشعبي منذ فترة طويلة لعلاج الالتهابات المختلفة لذا فان تصنيع مثل هكذا مواد حاجة حقيقية خصوصا في المستشفيات والمراكز الصحية.

المواد وطريقة العمل: استعملنا عدة تراكيز من مستخلصات الحبة السوداء المائي والكحولي (40،35،30،25،20،15،10)ملغم /مل ضد الفطر *Candida albicans*المعزول من تلاميذ المدارس الابتدائية المصابين. وقد تم دراسة تأثيره ومقارنتها مع النيستاتين باستعمال طريقة الانتشار بالاقراص.

النتائج: بينت النتائج أن المستخلص المائي ليس له تأثير تثبيطي، أما المستخلص الكحولي له تأثير معنوي في تثبيط الفطر. وأظهرت النتائج أن النستاتين له تأثير مثبط أكبر من تأثير المستخلص الكحولي لحبة السوداء المستعمل بمختلف التراكيز لغاية تركيز 40 ملغم/مل.

الاستنتاج: اثبتت الدراسة ان الحبة السوداء مصدرا للادوية المضادة للفطريات وكذلك في الطب البديل لعلاج الالتهابات المعوية الفطرية

The developing microbial resistance to the existing anti-microbial agents has become a real challenge and a serious problem[1]. *Nigella sativa (N.sativa)* is an annual flowering plant, native to southwest Asia. Among the promising medicinal plants, *N. sativa*, a dicotyledonous of the Ranunculaceae family, is an amazing herb with a rich historical and religious back ground [2,3,4]. *Nigella sativa* is found in southern Europe, northern Africa and Asia minor. The use of plants as medicines dates from the earliest years of mans evolution. Medicinal plants serve as therapeutic alternatives, safer choices, or in some cases, as the only effective treatment[3].

In Islam, it is regarded as one of the greatest forms of healing medicine available. Its most famous for the saying of the holy prophet Muhammad (peace be upon him) "Hold on to use of the black seed, for it has a remedy for every illness except death". The word "hold on to" indicate a long term use[5].

Nigella is known as kalonji (Hindi), habbat Al-barakah or habbat Al-Sauda (Arabic), kalvanji (urdu), siyah daneh (Persian). In English language or in the west, it is called fennel flower or sometimes just referred to as *Nigella* or black seed or black cumin or black caraway[2,6,7]. Seeds of *N. sativa* are frequently used in folk medicine in the Middle East and some Asian countries for the promotion of good health and treatment of many ailments[4]. Recently, many biological activities of *N. sativa* extracts have been reported, including antifungal, antibacterial, antiparasitic, and antiviral[8]. It has been reported that *N. sativa* seeds or its extracts have anti-inflammatory[9] and antimicrobial property[1].

In most healthy people, *Candida albicans* was a harmless micro flora but, in immunocompromised patients, develops into an opportunistic pathogen that can cause life threatening disseminated infection[10]. Overgrowth of *Candida albicans* in the intestines is responsible for a yeast syndrome that results in symptoms such as fatigue, diarrhea, constipation, rectal itching, inflammatory bowel disease (IBD), food sensitivity, headache, mood swings, sinus congestion, depression, poor memory and concentration, and cravings for sweets.

Typical antifungal drugs that used in some cases of *Candida* infection such as nystatin, and azoles derivatives as Fluconazole and Ketoconazole. nystatin is first choice and the most important antifungal that used to treat gastrointestinal tract infected with *Candida albicans*, because it is not absorbed throughout the GIT and so typical effect will occur, but the development of resistance and the presence of adverse effects of these drugs are an emerging trend that may threaten their effectiveness[11].

Materials and Methods :

Nigella sativa: (Seed Viability Test):

The seeds were purchased from a local herb shop in Iraq. There were identified, cleaned to remove any debris, air dried and cultured in a petridishe having wet clean filter paper or gauze and incubated at (25±1)◦C in dark growth chamber for few days. The filter paper were always kept wet by adding enough water to moisten the paper but pour out any extra water and then calculated the percentage of growth %[12].

Preparation and Sterilization of Black Seed Extracts (Aqueous Extracts):

Aqueous extracts of *Nigella sativa* can be prepared depending on Riose route by dissolving 40 g of seeds (after cleaning it from debris) in 160 ml of sterile distill water in ratio 1:4 (wt:vol) by using electrical blender inside cool place to prevent losing of volatile oil, for about 1 hour then leaves at 4°C for 24 hour (in refrigerator). The extract were filtered by using many layers of gauze and centrifuged at 3000 rpm for 5 min then filtered by wattman filter paper No.1. The extract were dried by using lyophilizor that dried by cooling under low vacuum[13] .The dried extract were stored at 4°c until using.

Aqueous extracts can be sterile by using Membrane Filter 0.45u, that is make by dissolving 1 g of dried aqueous extract in 5 ml of sterile distil water, so we have 200 mg / ml extract concentrations as crude concentration, this extracts will be sterilized by filtering through Membrane Filter 0.45u (GEMA MEDICAL S.L. .Spain)to prevent passage of microbe through it and make this concentration as a crude, from it we can prepare other concentrations which using in research

Alcoholic Extracts:

Nigella sativa alcoholic extract can be prepared depending on Grand route by crushing 20 g of the seeds(after cleaning it from debris) and dissolving it in 200 ml of 95% ethanol as in ratio 1:10 (w:v)

inside a cool place inside by using blender for about 1 hour ,then put the mixture in refrigerator at 4°c for 24 hour .The extract were filtered using many layers of gauze then centrifuged at 3000 rpm for 5 min and the filtrate were filtered through wattman filter paper no.1. The alcoholic filtrate was evaporated using rotary vacuum evaporator (Rota vapor R11:BUCHI)that evaporate the solvent under low vacuum and under 40°C temp to prevent losing the volatile oil which is the main active ingredients present. The filtrate will be further dried by using lyophilizor which dry the filtrate by cooling under low pressure[14]. The alcoholic extract were kept in refrigerator at 4°C until further testing .

For making inhibitory experiments, we should make sterilization for extracts and preparing different concentrations by using crude concentration and that is occur by weight 1 g of dried alcoholic extract and dissolve it in 5 ml of dissolvent like DEE(Diethyl Ether). then sterile the mixture by pasteurization method (heating in the water bath) under 62°C for 10 min.. So we prepare 200 mg / ml as a crude concentration which using for preparing other concentrations.

Preparation of Different *N. sativa* Extracts Concentration:

Different concentrations of *N. sativa* (aqueous and alcohol) extracts were prepared starting from (10, 15, 20, 25, 30, 35, 40) mg /ml in sterile distil water and Diethyl ether as a diluents respectively. Whatman filter paper No. 1 was used to prepare discs by cutting the paper in a diameter equal to 6 mm. The discs were then sterilized by autoclaving at 121°C for 15 min in tightly closed container having 10 discs. Adding 0.1 ml of different concentrations were prepared to each container and were dried in the bio safety cabinet for 15 minute. Negative control disc were prepared using DEE and DW. Positive control disc were prepared using nystatin drug[15].

Preparation of Antifungal (nystatin) Discs:

Depending on B.V.T.C.1979(Bacteriology Virology Tissue Culture), we prepare wattman no.1 filter paper discs containing 100 I.U. of nystatin (samara) on each disc, which is equal to 0.06 mg/disc (each 1 mg equal to 1666 I.U.). 100 IU of nystatin / disc is the international concentration that used in *C. albicans* sensitivity test against nystatin .By taking 1 tablet of nystatin that contain 500,000 I.U.and dissolve in 100 ml of DW to prepare 5000 I.U/disc of nystatin suspension ,then add 50 of sterile whatmann filter paper discs about 6 mm in diameter to 1 ml of the suspension, so nystatin disc on conc. of 100 I.U. / disc were prepared which is the desired conc. All the experiment should occur in sterile condition[16].

Collection of samples:

Stool samples were collected from students of primary schools: (Al-Arqam primary school for boys, Al-hady primary school for girls, and Al-Arpachia primary school for boys and girls) of about 6-12 years old. Stools were taken from students in a clean water-proof with a tight fitting container 10 ml saline as a transport medium[17]. The containers were labeled with patient's full name, age, sex and time of collection. Samples were examined in the laboratory within 24 hrs of collection. Each samples was transported at 37°C and examined directly under the microscope for yeast cell. By taking about 1 gm of the stool of each samples and cultured on nutrient broth and on sterile petridish containing Sabouraud dextrose agar (SDA) (having chloramphenicol and gentamicin as antibiotic to prevent contamination). *C. albicans* appears as large, round, white or cream colonies on agar plate . Further tests can be done for identifying *C. albicans* such as gram stain, germ tube (GT) formation in animal or human serum[18] and growth in corn meal agar (CMA) that form chlamydospore and pseudomycelium[19,20].

Inhibitory Effect of Aqueous Extract of *Nigella sativa* on *Candida albicans:*

To detect the inhibitory effect of aqueous extract of *N. sativa* on *C. albicans* using disc diffusion method by preparing different concentration of about 10, 15, 20, 25, 30, 35, 40 mg / ml from the crude concentration after sterilization using sterile distiled water as a diluents[6,7].

Inoculums were prepared using nutrient broth with 3-5 colonies of *C. albicans* from SDA. The inoculums were incubated at 37°C for 24 hours to get a suspension of about 415×10^5 cell /ml by using a chamber. SDA were cultured with

an inoculums of *C. albicans* using sterile cotton swabs that diffuse 0.1 ml of the *C. albicans* suspension on the surface of the agar. Whatmann no. 1 paper discs impregnated with different N.sativa aqueous extract conc. were carefully placed on the seeded plate and adding a negative control (disc impregnated in sterile D. W. only)in three repeated petridishs. The plates were incubated at 37±1°C and examined for zones of inhibition after 24 hours[21].

Inhibitory Effect of Alcoholic Extract of *Nigella sativa* on *Candida albicans:*

To detect the inhibitory effect of alcoholic extract of *N. sativa* on *C. albicans* using disc diffusion method by preparing different concentration of about (10, 15, 20, 25, 30, 35, 40) mg/ml from the crude conc. after sterilization (using DEE as a diluents) by dipping 10 sterile filter paper discs in 0.1 ml of each conc. of the extract[6,7].

Inoculums were prepared using nutrient broth with 3-5 colonies of *C. albicans* from SDA. The inoculums were incubated at 37°C for 24 hours to get a suspension of about 415×10^5 cell /ml by using a chamber. SDA were cultured with an inoculums of *C. albicans* using sterile cotton swabs that diffuse 0.1 ml of the *C. albicans* suspension on the surface of the agar. Whatmann no. 1 paper discs impregnated with different *N. sativa* Alcoholic extract conc. were carefully placed on the seeded plate and adding a negative control (discs impregnated in sterile D. W. and DEE)in three repeated petridishes. The plates were incubated at 37±1°C and examined for zones of inhibition after 24 hours[2].

Results:

Seed Viability Test:

Nigella sativa seed were purchased from a local herb shop in Iraq. Three samples were taken with different viability 67%, 93%, and 95%, depending on the source of purchasing and storage environment. The favourable one is the highest viability test

Extraction and Sterilization of *Nigella sativa*:

N. sativa alcoholic extract was in liquid form due to the presence of large amount of oil in the extract and appeared brown in colour. While aqueous extract of *N. sativa* was in gelatinous form, dark brown in colour, After lyophilization, it was not converted to powder form due to the presence of oil in the extract that aid to make the extract in high viscous gelatinous form.

Isolation and Identification of *Candida albican:*

Candida albicans isolated from 252 stool samples which appear as yeast cell that is small round form as present in Figure (1, 2,3).

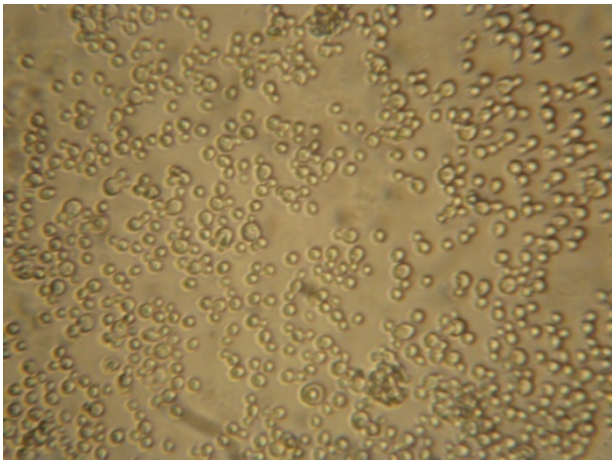


Figure (1)*Candida albicans* in a characteristic yeast cell with budding appeared on nutrient broth (40X).

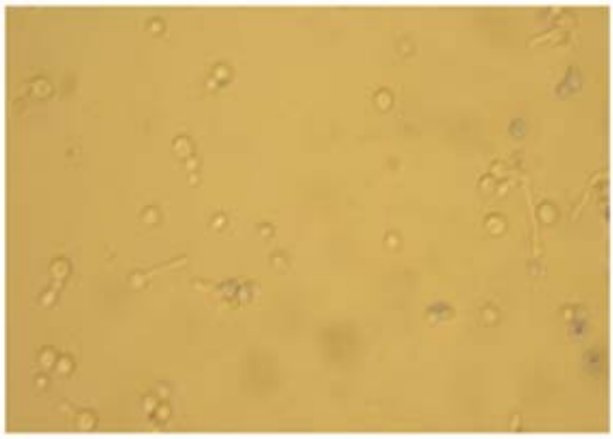


Figure (2): *Candida albicans* germ tube on serum at 37◦C for 2 hours(40X).

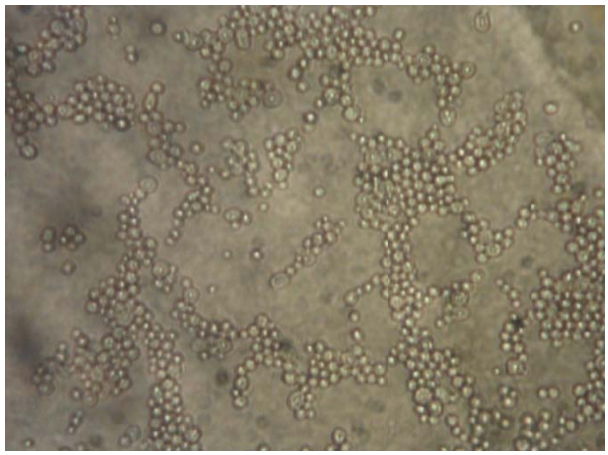


Figure (3):*Candida albicans* chlamydospore on CMA at 27◦C for 48 hours(40X).

Efficacy of *N. sativa* Extract on isolated *C. albicans:*

There were no effect of aqueous extract of *N. sativa* on *C. albicans* isolate, while the inhibition zone was 9 when 10 mg /ml concentration of *N. sativa* alcoholic extract was used and the inhibition zone was 17.3 when 40 mg /ml concentration of *N. sativa* alcoholic extract used. This means the increase in the concentration of alcoholic extract lead to further increase in inhibition zone as in Table (1) and Figure (4).

Table (1): The effect of alcoholic extract of *Nigella sativa* and nystatin on *Candida albicans* isolated from stool sample.

Type of material used	Conc. of the extract (mg⁄ml)	Average diameter of the inhibition zone (mm)
Alcohol extract of *N. sativa*	10	9
	15	9.8
	20	13.1
	25	13.7
	30	14.3
	35	15.3
	40	17.3
Nystatin	100 IU (i.e. 0.06 mg⁄ disc)	17.7

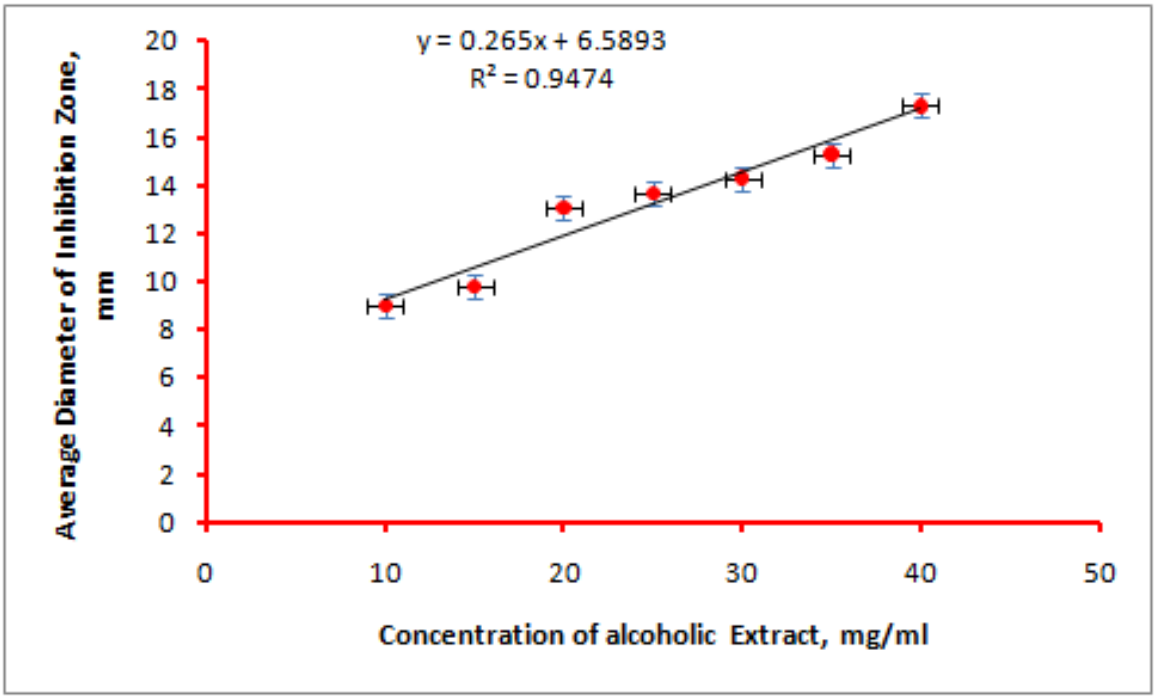
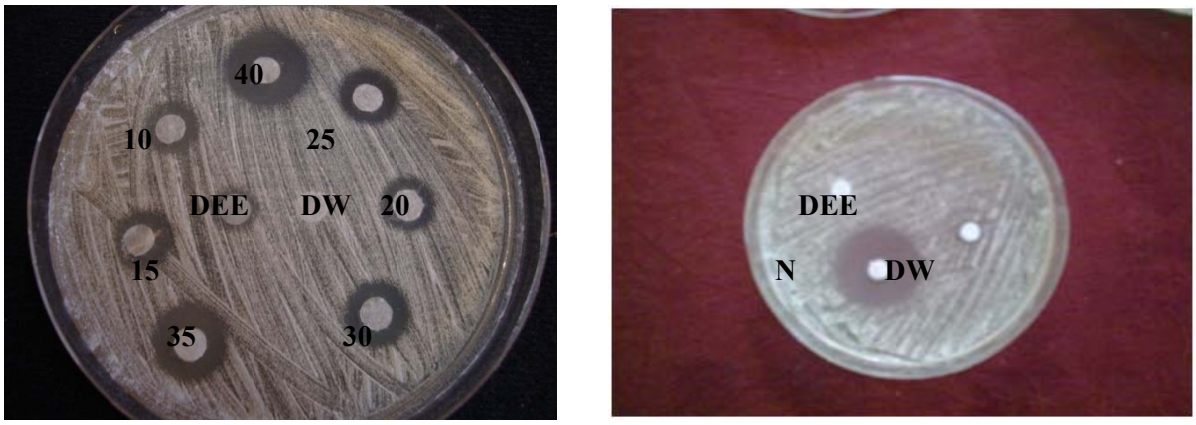


Figure (4): Comparison between the effect of alcoholic extract of *Nigella sativa* and nystatin on *Candida albicans* isolated from stool sample.



a **b**

Figure (5):(a) the effect of alcoholic extract of *Nigella sativa* on *Candida albicans* isolated from stool sample.(b) comparism between the effect of nystatin and negative control(distill water and diethyl ether) on *Candida albicans* isolated from stool sample.

Comparison Between *N. sativa* Extracts and nystatin on *C. albicans*

The inhibition zone of nystatin disc was more than that of alcoholic extract of *N. sativa* in concentrations used of about (10, 15, 20, 25, 30, 35, 40 mg⁄ml). The diameter of inhibition zone of discs treated with nystatin was 17.7 mm when treated with 100 IU (i.e. 0.06 mg⁄disc), while the diameter of inhibition zone of discs treated with *N. sativa* alcoholic extract was 9mm when treated with 10 mg⁄ml and increased to reach 17.3 mm when treated with 40 mg⁄ml show figure (5 a and b).

Discussion:

In the present study, *Nigella sativa* alcoholic extract was in liquid form due to the presence of large amount of oil in the extract and it appears brown in colour. The method of extraction in this work was depended on Grand route (22) which was modified from Verpoorte e*t al.*[22].

Nigella sativa aqueous extract was in high viscous gelatinous form. The method of extraction in this work was conducted to the procedure of El Wakil H.S.[23], Al-Heali F.M, and Rahemo Z[24] and Tonkal A.M.D.[9].

C. albicans samples were isolated from the stool of primary school students in a disposable clean dried container. *C. albicans* had been identified by several testing e.g. gram staining of the colonies showed large gram positive organisms suspected to be yeasts cells, formation of germ tube in animal or human serum[25] and chlamydospore and pseudomycelium in corn meal agar[26].

Inhibition zone diameter of each concentration of *Nigella sativa* of different extract has been measured. The effect of *N. sativa* aqueous and alcoholic extract on *C. albicans* was estimated throughout a series of concentrations (10, 15, 20, 25, 30, 35, 40 mg⁄ml) which were used. The study found that there was week effect of aqueous extract of *N. sativa* on *C. albicans* isolated, but there was significant differences in the inhibitory effect of *N. sativa* alcoholic extract on *C. albicans* isolated from stool sample and the inhibition zone increased as the concentration of the extracts increased. The inhibition zone was 9 mm when 10 mg⁄ml *N. sativa* alcoholic extract was used and the inhibition zone was 17.3mm when 40 mg⁄ml used. The study illustrated that 40 mg⁄ml had biggest inhibition zone while the concentration 10 mg⁄ml had the smallest effect as in table (1) and figure (4).

Absence of effect of aqueous extract was agreed with Abu-Al-Basal 2009[1], while disagreed

with Hanafy and Hatem 1991[27] and Al-Assaaf 2008[21]. The presence of inhibitory effect of alcoholic extract was agreed with Hanafy and Hatem 1991[27] and Al-Assaaf 2008[21], while disagreed with Abu -Al-Basal 2009[1].

To comparison between the inhibitory effect of *N. sativa* alcoholic extract and nystatin on *Candida albicans* isolate presented in table (1) and figure (5). The inhibition zone of nystatin disc were more than that of alcoholic extract of *N. sativa* in concentrations used of about (10, 15, 20, 25, 30, 35, 40 mg/ml). The diameter of inhibition zone of discs treated with nystatin was 17.7 mm when treated with 100 IU (i.e. 0.06 mg /disc), while the diameter of inhibition zone of discs treated with *N. sativa* alcoholic extract was 9 mm when treated with 10 mg / ml and increased to reach 17.3 mm when treated with 40 mg / ml. It had been indicated that the effect of nystatin on *Candida albicans* isolated were higher than that of *Nigella sativa* on concentration 40 mg / ml. So increasing the concentration may lead to higher effect than that of nystatin. Further study should be performed on this line is needed.

N. sativa extracts were proved to have an immunomodulatory effect..as they have a prominent stimulatory effect on CD4 positive T-cells and macrophages causing an immunomodulatory effect both in humans and animals[28]. However, their exact mechanism of action on the individual components of the immune system needs to be deeply investigated. Understanding of such mechanisms will put a great impact on the management of many infectious as well as immunological disorders.

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