Chemical composition and antimicrobial activity of Thymoquinone (Nigella sativa) against pathogenic bacteria in food products (cake)

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Abstract

Thymoquinone (2-Isopropyle-5-methyle-1, 4 benzoquinone) is well known for its broad-spectrum antimicrobial activity against a wide range of microorganisms. This study aims to determine the chemical composition and investigate the antimicrobial activity of cold pressed (CO) and essential oil (EO) of *Nigella Sativa* (Thymoquinone).Three concentration of thymoquinone (5%, 10%, and 15%) were tested against gram positive-gram negative bacteria (Staphylococcus aureus, Escherichia coli, Bacillus cereus ,Salmonella), Total coliform bacteria, and food born fungi (Aspergillus flavus and Candida albicans) in food products (Cake) storage foe 45 days at refrigeration temperature (4^0C).Gas chromatography-mass spectrometry (GC-MS) analysis revealed that the major component in Nigella sativa essential oils was thymoquinone (9.87%) followed by p-cymene (6.07%) .The results showed that thymoquinone exhibited the growth of all tested bacteria and fungi, its antimicrobial efficacy gradually with higher concentration during storage.

Introduction

The prevention of food borne diseases has led to the development of sophisticated food safety and control systems, mainly in developed countries. However, worldwide, the majority of countries acknowledge that food borne disease continue to be a major public health issue. Severe implications for both the health of individuals and the development of modern society can arise from food borne diseases. This fact is recognized by the World Health Organization (WHO) (WHO, 2000) and claims from each member state allowed for the development of systems to ensure a real reduction in the burden of food borne disease. Since ancient times, the antimicrobial impact of essential oils and their components isolated from aromatic and medicinal plants has been recognized for both health brnefits and food preservation. In the last decades these properties have been confirmed. The control of food spoilage and pathogenic microorganisms is mainly achieved though chemical control. However, the use of synthetic chemicals is limited due to undesirable aspects such as carcinogenicity, teratogenicity, acute toxicity, and prolonged degradation periods, which contribute to environmental pollution. The growing awareness among modern consumers about these issues has led toa demand for “green” food preservation methods, minimizing the use of synthetic

chemicals while ensuring extended shelf life. This has prompted the scientific community, agro-industries and pharmaceutical industries to search for natural compounds that meet consumer demands. Black cumin (Nigella sativa L.) belonging to family Ranunculaceae,has been for decades for both culinary and medicinal purposes. It is also recognized as a natural remedy for asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness, and influenza (Kabara *et al*., 2010). The seeds are known to be carminative, stimulant, and diuretic (Shah *et al.*, 2003). The essential oil from the seeds of this herbaceous plant contain high concentrations of thymoquinone and related compounds such as thymol and dithymoquinone, which have been implicated the preventing inflammation (Tekeoglu *et al*., 2006) and exhibiting antioxidant activities (Kruk *et al*., 2000).These include quenching reactive oxygen species, antimicrobial activity (Singh *et al*., 2006), and anticarcinogenic and antiulcer activity.

Material and method

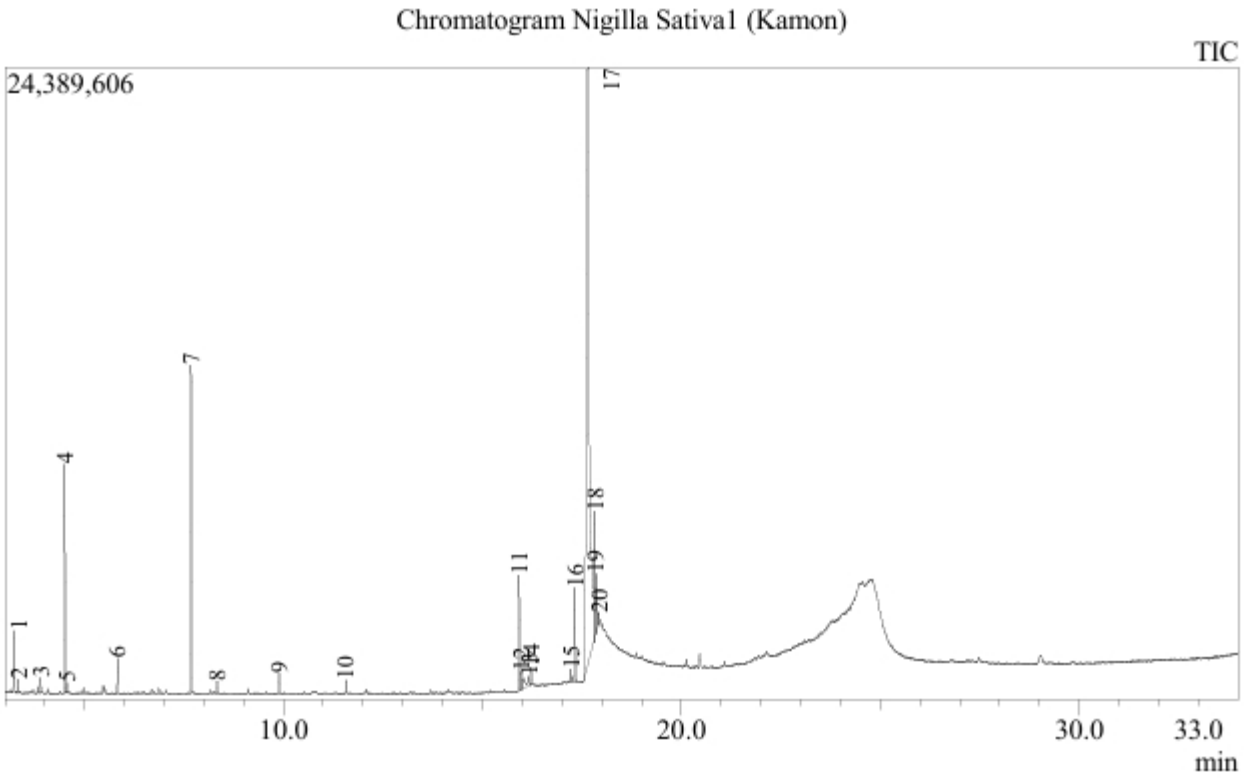
Extraction of black cumin oil:

A 2 kg sample of cumin powder was mixed with 4 liters of hexane and placed in a shaker for 36 hours. The mixture was then filtered through filter paper. The oil was separated from the hexane solvent using rotary evaporator 50^0C. The sample was stored in a dark glass container in freezer until analysis.

Gas chromatography_ Mass spectrometer

The chemical composition for the extracted oil was examined using GC-MS analysis, performed using a Shimadzu GC-2021 system coupled with a Shimadzu GCMS-QP2021 Ultra network mass _selective detector, equipped with DB-1MS capillary fused silica column (30m ,0.25mm I.D.,0.25µm film thickness). A1µL cumin oil solution in Diethyl ether (HPLC grade) was injected and analyzed. The column was initially held at 40°C for 5 minutes, then increased to 250° C at a rate of 4°C /min, and held at 250° C for 1 minute. Other operating conditions were as follows: carrier gas, He (99.999%); with a flow rate of 1.82 ml/min: injector temperature, 250 C°; split ratio, 1:50. Mass spectra were taken at 70ev. Mass range was from m/z 35-50 amp. The relative percentage amounts of the separated

compounds were calculated from total ion chromatograms using a computerized library literature data (Fug 1).



(Fug 1) GC-MS Chromatography analysis of Nigella sativa

Peak#	Name	R.Time	Area	Area%
1	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	3.222	3091506	1.63
2	.alpha.-Pinene	3.331	723183	0.38
3	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	3.894	723337	0.38
4	o-Cymene	4.498	11521761	6.07
5	D-Limonene	4.559	502151	0.26
6	(1R,4R,5S)-1-Isopropyl-4-methoxy-4-methylbicyclo[3.1.0]hexane	5.834	1906898	1.00
7	Thymoquinone	7.678	18748762	9.87
8	Phenol, 2-methyl-5-(1-methylethyl)-	8.329	703715	0.37
9	Longifolene	9.888	1263208	0.67
10	p-Cymene-2,5-diol	11.568	749749	0.39
11	n-Hexadecanoic acid	15.922	9601028	5.06
12	Z-8-Tetradecen-1-yl acetate	15.983	1347254	0.71
13	Citronellyl butyrate	16.159	2471494	1.30
14	Hexadecanoic acid, ethyl ester	16.226	1023975	0.54
15	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.211	798648	0.42
16	Widdrol hydroxyether	17.324	5383243	2.83
17	9,12-Octadecadienoic acid (Z,Z)-	17.646	114084548	60.08
18	Linoleic acid ethyl ester	17.822	10505224	5.53
19	Ethyl Oleate	17.864	4316305	2.27
20	Z,E-2-Methyl-3,13-octadecadien-1-ol	17.917	436919	0.23
			189902908	100.00

Table (1) The Chemical Composition of Nigella Sativa extracted oil

Preparation of composite flour blends:

Cake standerd formula:750g flour, 200g milk, 200g powdered sugar,4 eggs, 10g Baking powder, 1g salt, 10 ml liquid vanilla, and 200ml oil (with addition thymoquinone).The ingredients were mixed for 3-4 minutes and placed on aluminum plates, then backed in an oven at 160^0C for 30 minutes, cooking foil was also placed in the oven for sterilization. After baking, the plates were covered with sterile cooking foil, cut into 5×5 cm square pieces, and packed in polypropylene films to prevent drying before analysis. Cakes were prepared with five samples variations: The first sample without BUT (control A).The second sample was prepared with addition of 200 ppm BUT as synthetic antioxidant (control B). While other sample were prepared by addition thymoquinone at three different concentration (5%, 10% and 15%) as natural antimicrobial.

Microbial Analysis:

Total valuable count of bacteria:

The pour plate count method (Harrigan, 1998) was used with plate count agar as the medium.

Preparation of Serial Dilution:

A10 ml of sample was homogenized in 90 ml of sterile diluents (0.1 Peptone solution).This gave a 10^{-1} dilution, and sterile dilutions continued until 10^{-6}. one ml from each dilution was transferred into sterile Petri dishes, mixed with 15 ml of sterile melted plate count agar, and allowed to solidify. Plates were incubated at 37^0C for 48 hours. A colony counter was used to count the viable bacterial colonies after incubation, and the results were expressed as colony-Forming Units (CFU/gram). (Harrigan, 1998).

Determination of coliform bacteria:

It was carried out by using the Most Probable Number (MPN) techniques.

Presumptive coliform test:

one ml of each of the three first dilution (10^{-0}, 10^{-2}, and 10^{-3}) was inoculated in triplicate into MacConkey Broth tubes containing Durham tubes. The tubes were incubated at 37^0C for 48 Hours. The production of acid with sufficient gas to full the concave part of the Durham tube was considered a positive presumptive test.

Confirmed test for total coliform:

Form every tube showing positive result at tube of Brilliant green 2% Bile Broth was inoculation was performed using sterile loop. The tubes were incubated at $37C^0$for 48 hours.

Confirmed E. coli Test:

Form every tube showing positive result in the presumptive test, inoculate a tube of EC broth containing a Durham tube. The tubes were incubated at 44.5^0C for 48 hours. Tubes showing any amount of gas were considered positive. Then, the Most Probable Number (MPN) was recorded.

For further confirmation of E. coli tube of EC broth which showing a positive result were streaked on Eosin Methylene Blue (EMB) agar. The plate were

incubated at 37^0C for 48 hours. Colonies of E. coli are usually small with metallic green sheen on EMB agar.

Detection of salmonella:

Twenty -five grams were weighed aseptically from the sample and homogenized in 225 ml of peptone water, and incubated for 24 hours at 37^0C. At the end of the incubation period, a 10 ml mixture transferred and incubated in 1000 ml of selenite cysteine broth, which was incubated for 24 hours at 37^0C. Aloopful of the 24- hour inoculum was streaked on plate of bismuth sulfite agar surface. It was incubated at 37^0C for 24-72 hours, black metallic sheen discrete colonies indicated the presence of salmonella.

Antimicrobial activity

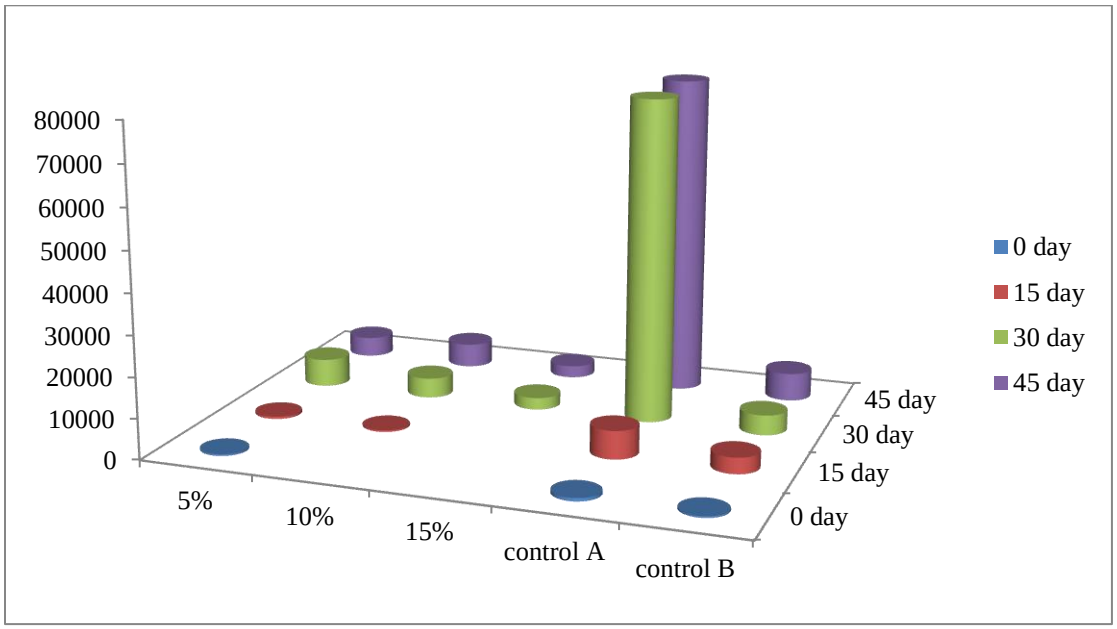
The antibacterial activity of the extracted oil was assayed by using bacteria, the microbial concentration 106 CFU/ml and by Well-diffusion method on Muller-Hinton agar, and the inhibition zone was measured in millimeter (mm).all Petri dishes were used as an experimental unit and the trial was repeated twice. Cultures were incubate at 37 C° for 24 hours. Similarly, the antifungal activity of the extracted oil was tested against fungal isolates [*Aspergillus flavus*, *Candida albicans*, and by using Sabouraud Dextrose agar medium, three Petri dishes were used as an experimental unit and the trial is repeated twice. The fungal cultures were incubate at 27 C° for three days , are shown in table 2 and picture 1.

Clinical isolates of bacteria	Inhibition zones (mm)
Bacillus cereus	52.4
Staphylococcus aureus	47.9
Salmonella	48.5
Escherichia coli	55.4
Candida	53.2
Aspergills	51.2

Table (2) A antibacterial and antifungal activity of Nigella Sativa extracted oil against the clinical isolated of bacteria and fungi

Total viable count of bacteria (CFU/g)

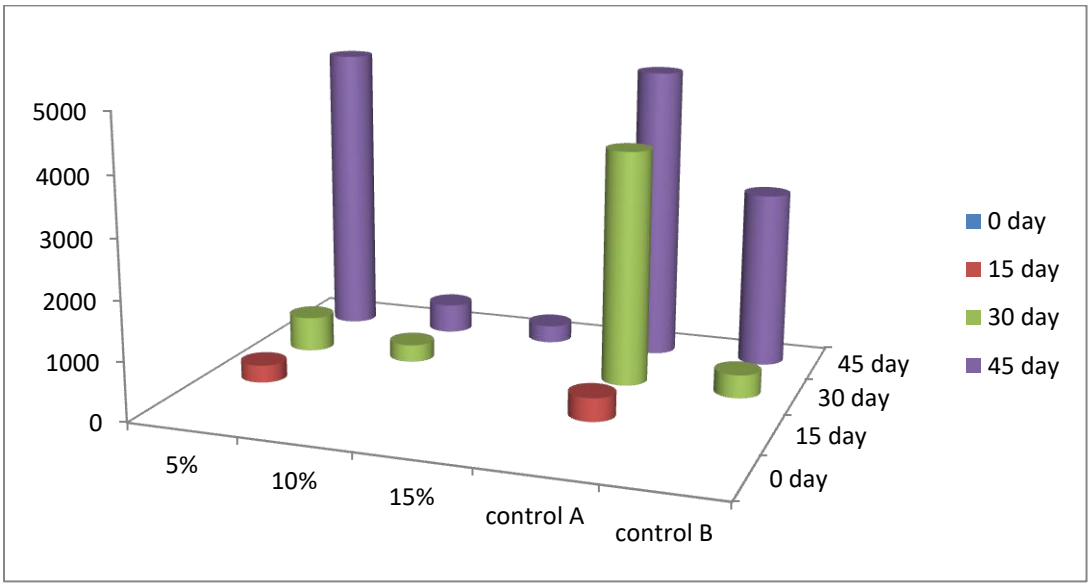
It was observed that a 5% concentration increases the total viable count of bacterial (CFU/g) during storage days: $3.0x10^2$, $8.0x10^2$, $5.0x10^3$, $7.5x10^3$ and $8.0x10^4$ respectively. Meanwhile, the sample with 10% concentration of thymoquinone showed no detectable presence of bacterial on the first few days, However bacteria appeared after 15 days of storage and increased over time: $3.0x10^2$, $5.2x10^3$ and $6.3x10^4$. The 15% concentration showed the best results, as no bacteria were detected on first day or after 15 days of storage. The total viable count of bacteria appeared only after 30 and 45days of storage and was minimal: $3.0x10^3$and $3.2x10^4$ respectively. Additionally, Control A (without the addition any antimicrobial agent) recorded a high total viable bacteria count, particularly after 30 and 45 days: $8.0x10^2$, $7.4x10^3$, $8.8x10^4$ and $8.9x10^5$.On the other hand control B (with the addition of BUT) showed lower results compared to control A and the 5% concentration with equal results to the 10% concentration except for a slight increase after 45 days of storage: $5.0x10^2$,$4.0x10^3$,$5.8x10^3$and $7.5x10^4$ (figure 2).



(Figure 2) Total viable count of bacteria cfu/g

Total Count of Yeast (cfu/g)

None of the samples showed any yeast growth initially. However, after 15 days of storage, yeast growth appeared in the 5% concentration sample and controls A ($4.0x10^2$ and $4.7x10^3$ cfu/g respectively). The 15% concentration of thymoquinone exhibited the best results, with no yeast growth observed until after 45 days of storage ($3.0x10^2$ cfu/g).In comparison, the yeast count in control A increased to $5.0x10^2$,$4.3x10^3$ and $5.6x10^3$ cfu/g while control B showed counts of $4.0x10^2$ and $6.3x10^3$ cfu/g (figure 3).

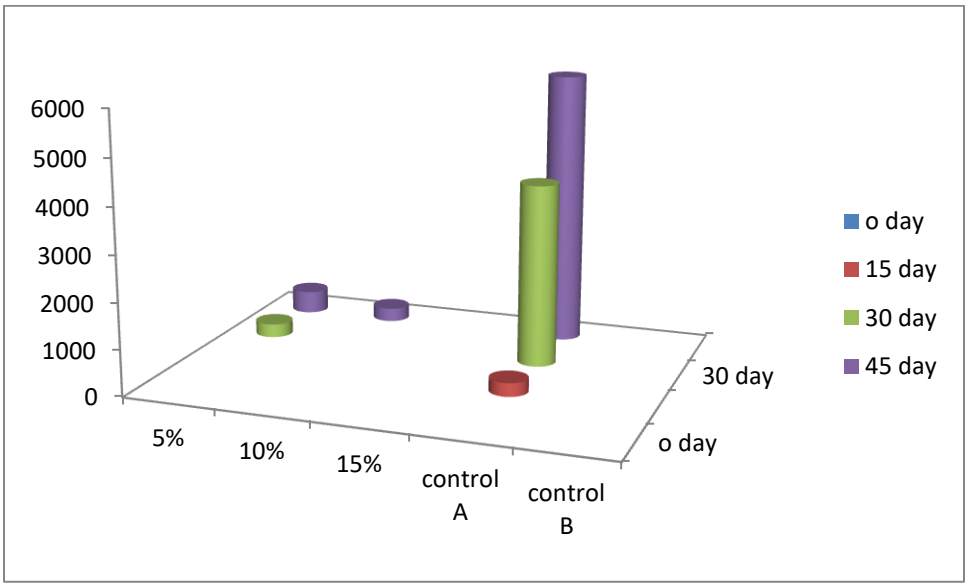


(Figure 3) Total count of yeast during storage

Total count of mold (Candida)

The experimental results show no candida growth in samples with 15% concentration and control B. Meanwhile, candida did not appear in the 10% concentration sample until after 45 days of storage ($3.0x10^2$cfu/g). In contrast, candida growth was observed in the 5% concentration sample candida after 30 and

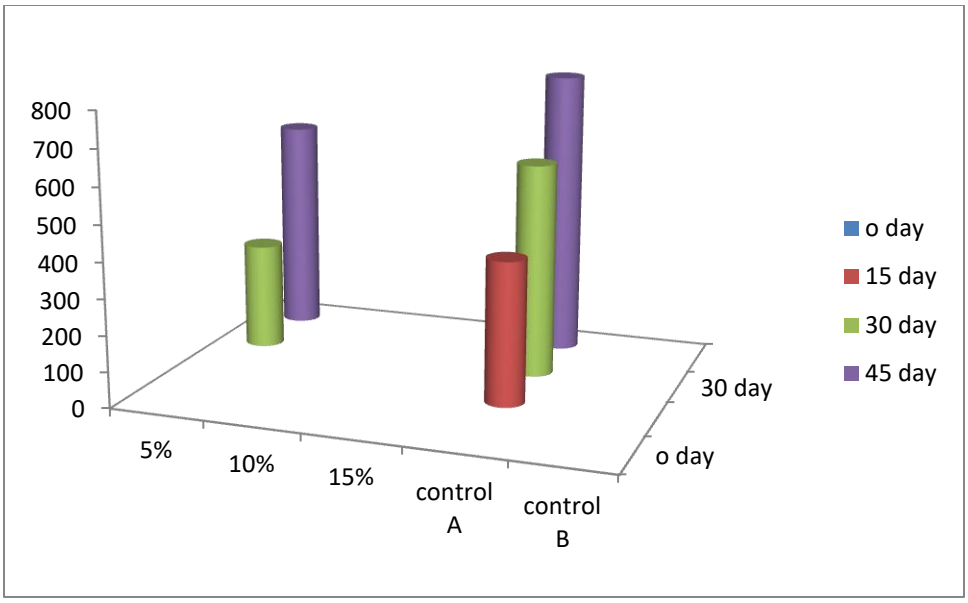
45 days of storage ($3.0x10^2$, 4.0x102cfu/g, respectively) ($p \geq 0.05$). Control A recorded the worst results, with candida appearing after 15 days of storage and increasing to $3.0x10^2$, $3.2x10^3$and $7.0x10^3$ cfu/g respectively (figure 4).



(Figure 4) Total count of candida

Total Count of Staphylococcus aureus

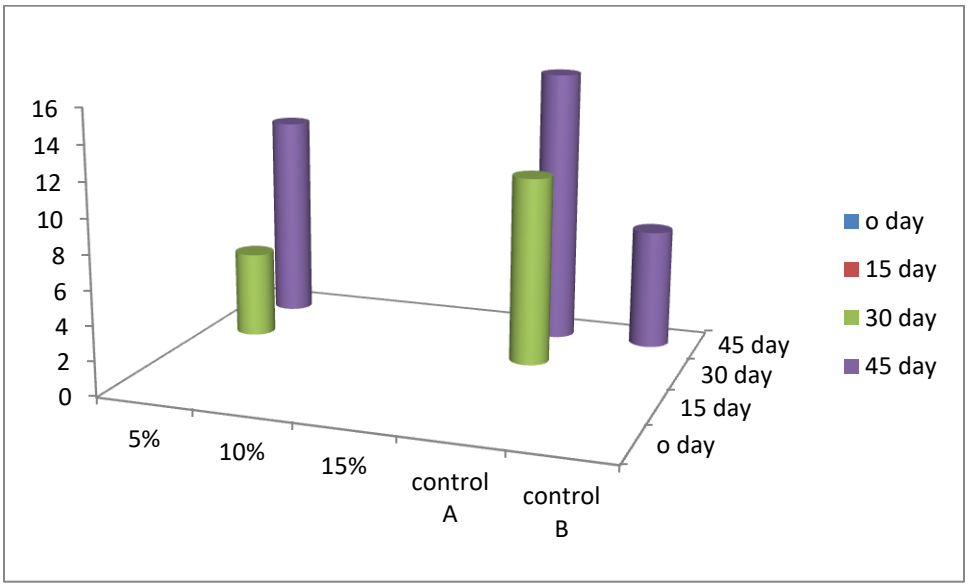
No growth of staphylococcus aureus bacteria was observed in samples with 10%, 15% and control B. Meanwhile, the 5% sample showed bacterial growth after 30 and 45 days of storage ($3.0x10^2$, $5.0x10^2$cfu/g, respectively). Control A exhibited the worst results, with bacterial growth appearing after 15 days of storage. The number of bacterial colonies continued to increase with the extension of the storage period reaching $5.0x10^2$, $7.0x10^2$ and $9.0x10^2$ cfu/g. respectively (figure 5).



(Figure 5) Total count of staphylococcus aurous cfu/g

Total count of coliforms (MPN/g)

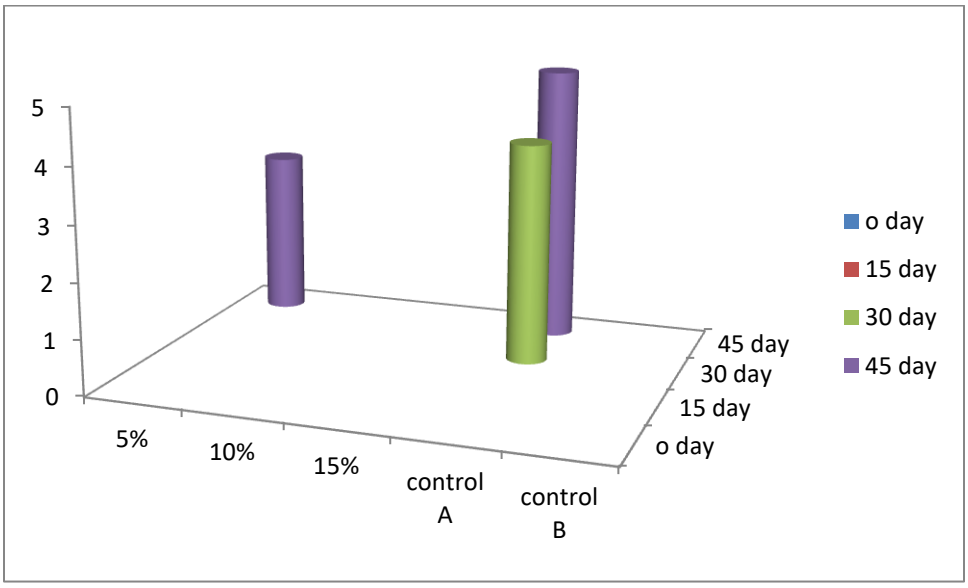
It was observed that no growth or presence of coliform bacteria was detected in samples with concentration of 10% and 15% during storage. However, control B bacteria growth did not occur until after 45 days of storage. In contrast, bacterial growth appeared earlier in control A and 5% concentration sample, where coliform bacterial were detected after 30 and 45 days of storage (figure 6).



(Figure 6) Total count of coliform MPN/g

Total account of Escherichia coli bacteria (MPN/g)

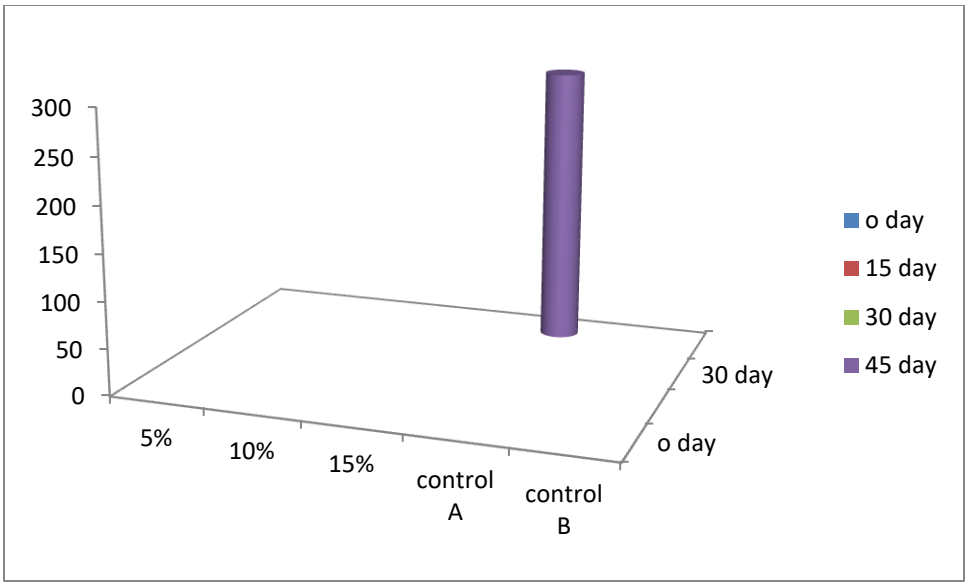
The analyses showed no presence of Escherichia coli bacteria in the samples with concentration 10% and 15%, as well as in control B, throughout the entire storage period. However, Escherichia coli bacteria appeared in the 5% concentration sample after 45 days of storage. In control A, they were detected after 30 and 45 days of storage (figure 7).



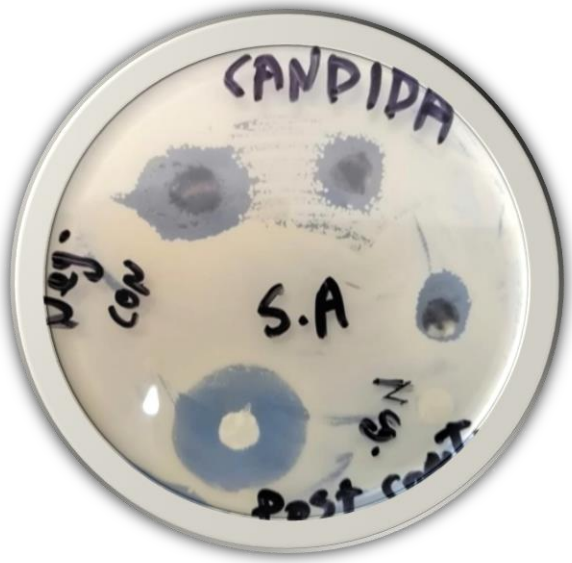
(Figure 7)Total count of Escherichia coli

Total count of bacillus cereus (cfu/g) and detection of salmonella

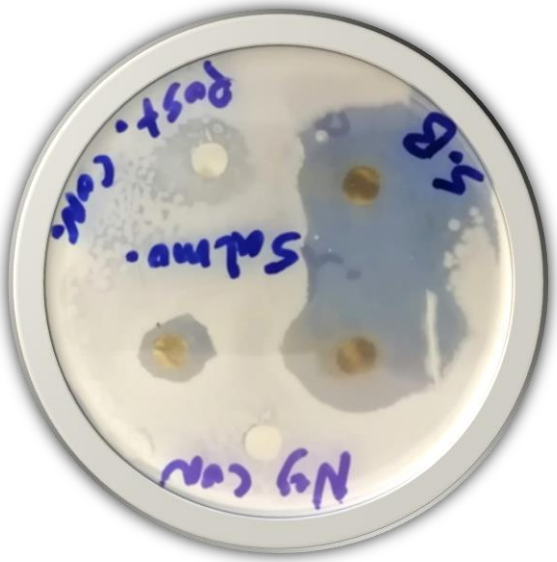
The test results showed that all samples were free from Bacillus cereus and salmonella during the storage period, except for the control sample A. In this sample, the growth of bacillus cereus and salmonella was observed after 45 days of storage (figure 8).



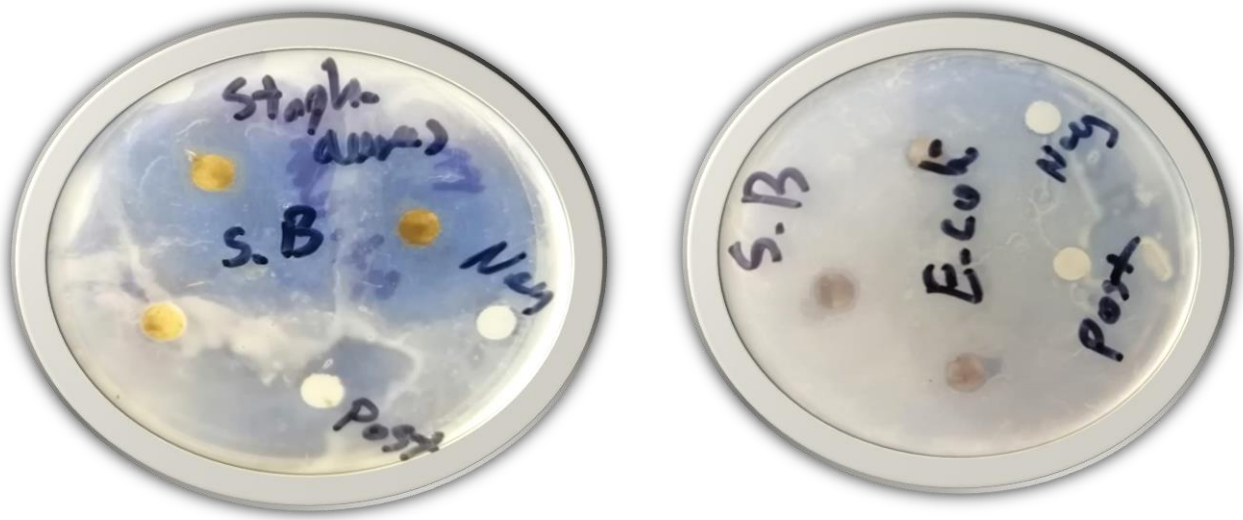
(Figure8) Total count of Bacillus cereus cfu/g and detection of salmonella



Candida



Salmonella



staphylococcus aurous **Escherichia Coli**

Picture (1) Antibacterial and Antifungal activity of thymoquinone

Discussion

Most of the antimicrobial activity in essential oils derived from spices and culinary herbs is believed to originate from phenolic compounds. Nigella sativa essential oil contains significant amounts of phenolic compounds (Thymoquinone) which might be responsible for its antimicrobial potential. The strength of inhibition and the spectrum of antimicrobial activity of N. sativa essential oil suggest that interactions between individual components lead to overall activity (Singh et al., 2005). Characterization of Nigella sativa essential oil composition by gas chromatography –mass spectrometry analysis has revealed the presence of a variety of compounds possessing antimicrobial properties, including thymoquinone, p-cymene y,terpinene and carvacerol (Gulluce *et al*.,2003; Gulluce *et al*.,2009; Suntres *et al* ., 2015; Oumzil *et al*,2006). Mahgoub et al (2013) studied the impact of adding N. sativa oil at levels of 0.1% and 0.2% (w/w) to Domiati cheese supplemented with probiotic cultures on the inhibition of food-borne pathogens, including Staphylococcus. aureus, Escherichia. coli, Listeria monocytogenes, and Salmonella enteritidis) inoculated in cheese during storage. Nigella sativa oil demonstrated antimicrobial activity, with a concentration of 0.2% exhibiting the highest antimicrobial potential against pathogens compared to with the control. The

storage life of oil-supplemented cheeses was extended under refrigerated conditions with low microbial loads. In addition, oil-supplemented Domiati cheese had also showed improved physicochemical and sensory properties. Some research found that Cumin oil blocked the growth of fungi and could completely stop fungal growth in modest concentration (Lacobellic et al 2005). Moreover, Cumin essential oil has exhibited antifungal activity against Aspergillus sp. and Candida albicans (Romagnoil et al 2010).

Conclusion

In this study we observed that cumin (N. sativa) possesses remarkable properties and has been extensively studied, it is widely used by people as a spice in their food, and its seeds are readily available in markets at a low price This study revealed thaT black cumin essential oil constitute a good alternative source of essential oil compared to common seed oil. Thymoquinone represents a promising natural alternative for combating food borne pathogens due to its antimicrobial properties and its ability to protect food from spoilage. With the growing interest in natural solutions and the need to reduce the use of synthetic preservations. thymoquinon can play a significant role in improve food safety and reducing food borne diseases.

Future application

Give its effectiveness as a natural antimicrobial agent, thymoquinone holds potential for various applications

Food preservation: thymoquinone can be added to food as a natural preservation. Preventing the growth of harmful bacteria and extending shelf life.

Pharmaceutical industry: Development of natural antimicrobial agents based on thymoquinone for treating infections caused by food borne pathogens.

Active packaging: Incorporating thymoquinone into active food packaging to inhibit microbial growth inside food containers.

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