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ORIGINAL RESEARCH article

Comparative composition, antioxidants, and antimicrobial effects of 3- and 7-day fermented seeds of *Nigella sativa*

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Keywords: Bioactive components, fermentation, GC-MS, *Nigella sativa, Staphylococcus aureus*

Abstract: The significance of fermentation is gaining more relevance due to the need for better preparation of plants, the desire for better plant constituents, and the aim for the preservation of medicinal plants. Hence, this study sets out to determine and compare the pH, proximate composition, chemical constituents, antioxidant, and antimicrobial effects of 3-day and 7-day fermented seeds of *Nigella sativa*. The fermentation of the seed was done for 3 and 7 days using 2.5% sugar. The pH of the sample declined from 3.5 to 3.45 during the fermentation. The proximate composition (moisture, ash, lipid, protein, and carbohydrates) was higher for the 3-day fermented sample while fiber was higher for the 7-day sample. The phytochemical constituents (tannins, saponins, phenols, alkaloids, flavonoids, phytates, oxalates, terpenoids, steroids, and glycosides) were higher for the 3-day than the 7-day sample. The Gas Chromatography-Mass spectrometry technique showed peaks that were characterized as 17 and 20 constituents on days 3 and 7 of the sample, respectively. The antioxidant capacity (ranging from 35.50% to 82.69% for the 3-day and from 37.50% to 84.99% for the 7-day sample) varies by the increasing concentration (7.81 mg/mL to 1000 mg/mL) of the sample. The diameter of zones of inhibition tested at different concentrations (25 mg/mL, 50 mg/mL, and 100 mg/mL) showed varying degrees of activity against *Proteus vulgaris* and *Staphylococcus aureus*. The zones obtained were higher 8-10 mg/mL against *Staphylococcus aureus* and 10-12 mg/mL against *Proteus vulgaris* for the 7-day fermented sample while 8.0 to 9.0 mg/mL was obtained for the 3-day fermented against *Staphylococcus aureus* and 8.0 to 10.0 mg/mL against *Proteus vulgaris*. Overall, the results of this study indicated that fermentation contributed to enhancing the bioactive components and antioxidant capacity of the fermented seeds of *N. sativa* thereby supporting the use of fermentation in the production of value-added functional foods.

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Introduction

Nigella sativa (*N. sativa*) seeds, commonly known as blackseeds, of the Ranunculaceae family, have been cultivated annually for use as food and medicine. Its nutritional and medicinal use dates back centuries in the Middle East, North Africa, the Far East, and Asia [1, 2]. Some scientific literature has reported numerous active ingredients of *N. sativa* such as thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellicine, and a-hederin [2, 3]. Ascribed to *N. sativa* are several properties as cellular protection [4], anti-inflammatory [5], antidiabetic [6], immune-stimulating [5], anticancer [7, 8], antimicrobial [1], and anti-rheumatoid arthritis [9] activities. Different extraction techniques involve the use of chemical solvents based on polarity, such as water [10], methanol, ethanol, ethyl acetate, chloroform, petroleum ether, n-hexane [11, 12], which are more or less harmful to human intake, however, fermentation mostly uses aqueous solvent which yields alcohols and acids as the end products and helps in better extraction of active compounds with no additional chemical solvents been added [13]. Therefore, fermentation has been classified as an environmentally safe method over other extraction methods. Since most of the phytochemical compounds naturally occurring in plants are bound and are less bioavailable than the free form, the goal of researchers focuses on achieving different approaches such as fermentation to improve on an existing phenomenon or generate a new one for preparing plants for enhancing their properties and promoting their medicinal value. While several pieces of literature have highlighted the significant and nutritional composition of black cumin extracts obtained using different solvents including methanol 14], and aqueous [15], there is little or no literature data on the fermented seeds of *N. sativa* and their chemical composition. Traditional fermentation, through the action of microbial enzymes, modifies foods and significantly imparts the qualities such as composition, flavour, aroma, texture, digestibility, and nutrition [16], medicinal [17] and combats the toxicity and anti-nutritive components of the food [18]. To promote the health benefits of plants, fermentation disintegrates the structure of foods to release compounds regarded as secondary metabolites [19] known for their medicinal values [20] and antioxidant capacities [21]. Hence, the goal of this study is to determine the phytochemical, proximate, antioxidant, and chemical composition of fermented seeds of *Nigella sativa*.

Materials and methods

Plant materials: Dried seeds of *N. sativa* were obtained from local vendors in Ilorin, Nigeria. The seeds were sorted to remove stones, worms, and dirt and were quickly rinsed with distilled water before use.

Preparation of the sample: Fermentation was done by a modified method of Hernández et al. [22]. Granulated sugar (20.0 g) was introduced into 100 mL of distilled water, a pack of yeast containing 7.0 g of yeast (Red Star® Quick-Rise) was added into the sugar solution, then 20.0 g of neatly picked and rinsed, with distilled water, black seeds were introduced into the mixture. The mixture was placed in a microwave set to 1.65 kw for 15 sec to fully activate the yeast at a resulting temperature of 43.0ºC. The cap was loosened to allow the carbon dioxide to escape. The mass of the reaction mixture was measured as a function of time. The jar mixture was kept at room temperature (26.0±2.0ºC). The cap was checked daily and the content was shaken to ensure the seeds were fully immersed in the solution After seven days, the sample was membrane-filtered (0.4 m) and refrigerated at -18.0ºC for further analysis. Sugar was added to raise the activity of yeast fermentation because it feeds on yeast enzymes, which convert sugar into alcohol and carbon dioxide [23].

A range of 100.0%, 50.0%, and 25.0% stock solution was prepared using distilled water via serial dilution method. From the test tube containing a 100.0% concentration of the sample, 10.0 mL was transferred into a sterile test

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tube containing 10.0 mL of distilled water, vortexed to mix well. Another 10.0 mL was transferred from this mixture into a fresh test tube containing 10.0 mL of distilled water to have 50.0% and 25.0% concentrations of the sample. The tubes were centrifuged at 3000 rpm for 20 min at 25°C and the supernatant was collected from each tube by filtration using Whatman no. 1 filter paper.

Proximate analysis: The moisture, protein, fat, crude fiber, ash, and carbohydrate contents of the fermented sample were determined with AOAC methods [24]. While the fat content was determined by the Soxhlet extraction method, the crude protein content was determined by the Kjeldahl method, and the carbohydrate was determined by weight difference: 100 (% moisture + % ash + % fat + % crude + % fibre + % crude protein).

Qualitative phytochemical screening: Phytochemical constituents of saponins, phenol, alkaloids, steroids, terpenoids, glycosides, and flavonoids were analyzed using the method described by Odebiyi and Sofowora [25].

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis: A mass of 10 g of the fermented sample solution was introduced into 30.0 mL ethanol overnight and filtered through ashless filter paper with sodium sulfate (2.0 g). The extract (which was believed to contain polar and nonpolar phytocomponents) was further concentrated to 1.0 ml by bubbling nitrogen into the solution. The ethanol extract of the fermented sample (2.0 µl) was employed for GC-MS analysis using a Clarus 436 GC Bruker which used a fused silica column BR-5MS (5.0% diphenyl/95.0% dimethyl polysiloxane), 30 m × 0.25 mm ID × 0.25 µm 2.0 µl df and the components were separated using Helium as carrier gas at a constant flow of 1.0 ml/min. The sample extract was then injected into the instrument which was detected by the Turbo gold mass detector (Perkin Elmer) with the aid of the software MS Workstation 8. The oven was maintained at a temperature of 110°C with 2 min holding during the 36th min of the GC extraction process. The injector temperature was set at 250°C (Mass Analyser). The different parameters involved in the operation of the Clarus 436 MS Bruker were also standardized (Inlet line temperature: 200°C). Mass spectra were taken at 70 eV; a scan interval of 0.5s and fragments from 45 to 450 Da. The MS detection was completed in 36 min [26].

Antioxidant activity: The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) technique was employed to assay for free radical scavenging activity of the fermented sample. This was achieved by measuring 500 µl of 0.3 mM alcoholic solution of DPPH and adding it to 2.5 mL of fermented samples at different concentrations (250-1000 µg/mL). The samples were incubated in the dark for 30 min, and a UV-visible spectrophotometer (Systronics AU-2700) was used to measure the absorbance at 518 nm. Synthetic antioxidant butylated hydroxytoluene (BHT) was used as a positive control [27]. The DPPH was assayed in triplicates and scavenging activity was given as percentage inhibition using the following formula: % Scavenging = $[(Abs_{control} - Abs_{samples})/ Abs_{control}] \times 100$

Antimicrobial activity: Antimicrobial assay of the fermented sample against *S. aureus and P. vulgaris* was performed by agar well diffusion method [28] using Mueller Hinton Agar. The viability of the test organisms was done by inoculating them in nutrient broth and incubating them overnight at 37°C. Colonies from the plates were transferred from the sub-cultured agar plate using a sterile inoculating loop into the sterile normal saline in a sterilized test tube. This was standardized using a UV spectrophotometer (Systronics AU-2700) to get an absorbance reading of 0.063-0.10 at a wavelength of 600 nm matching 0.50 McFarland turbidity standard giving a final inoculum of 1.5×10^8 CFU/mL [29]. Following this, 1.0 mL of the standardized inoculum was introduced using a pipette into the center of the molten MHA plate, swirled well, and allowed to solidify. An equivalence of 50.0 µL of the different concentrations of the fermented sample was added into the bored wells (6.0 mm). A positive control (amikacin 30 mcg) was added to a fresh well. The sample was allowed to stay for 30 min at room temperature for proper diffusion into the impregnated medium and incubated for 24 hrs at 37°C. After which, the

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plates were observed for clear zones around the well, the zone of inhibition was measured in mm and recorded as the antimicrobial activity of the sample against the tested pathogens.

Results

The pH value of the fermented seeds of *N. sativa* declined during days 3 and 7 of the fermentation period and was found to range from 3.5 to 3.45. The proximate composition of the 7-day fermented seeds of *N. sativa* with values of the proximate components (moisture, ash, lipid, protein, and carbohydrates) was higher on day 3 while fiber was higher on day seven (**Figure 1**). The phytochemical constituents (tannins, saponins, phenols, alkaloids, flavonoids, phytates, oxalates, terpenoids, steroids, and glycosides) were higher for the 3-day than the 7-day fermented seeds of *N. sativa* (**Figure 2)**.

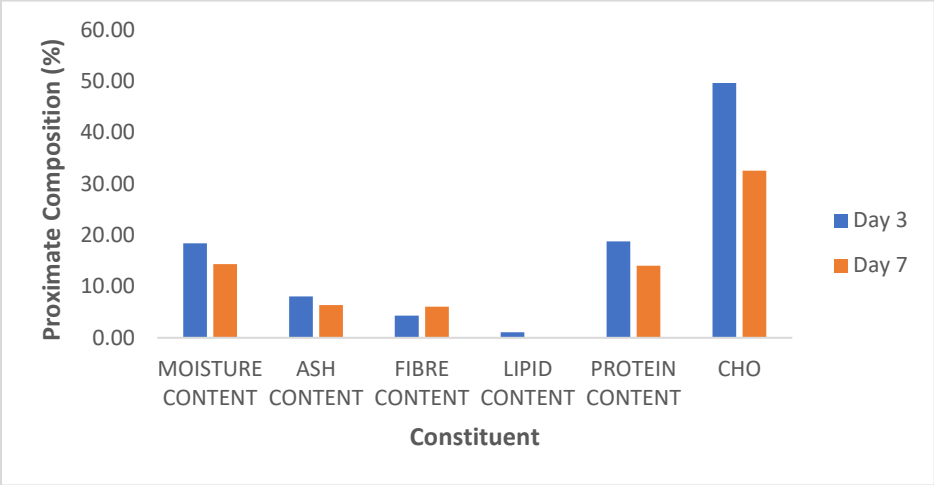

Figure 1: Proximate analysis of 3-day and 7-day fermented seeds of *Nigella sativa*

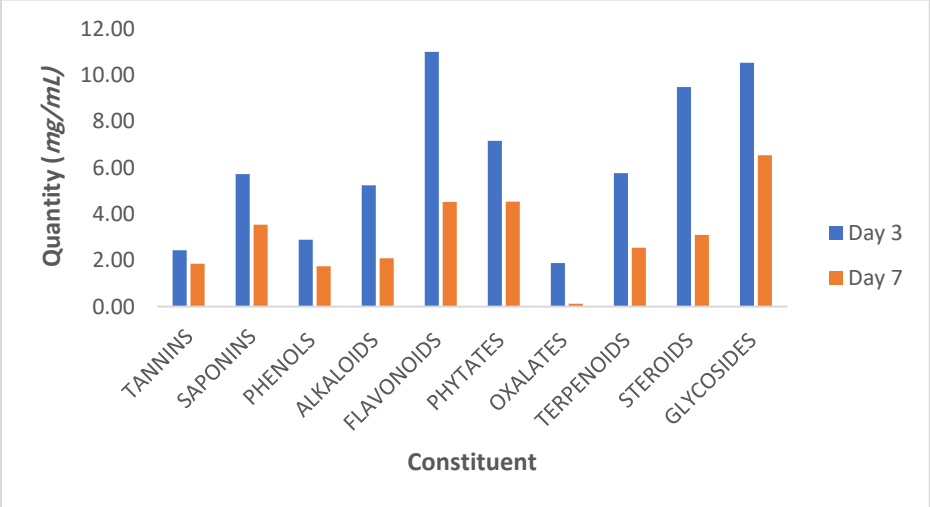

Figure 2: Qualitative phytochemical constituents of 3-day and 7-day fermented seed of *Nigella sativa*

Twenty compounds were identified in the 7-day fermented sample (**Table 1**) while nineteen compounds were identified in the 3-day fermented sample (**Table 2**) through mass spectrometry attached with Gas Chromatography relating to their peak area and retention time (**Figures 3** and **4**).

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Table 1: Compounds associated with the 7-day fermented seeds of *Nigella sativa* characterized by GC-MS

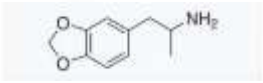
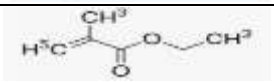
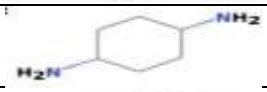
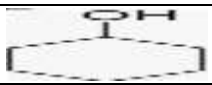
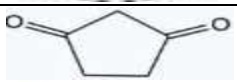
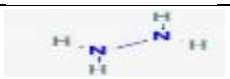
No	Rt (min)	Name of compound	Molecular formula	MW g/mol	Peak area	Structure of the compound
1	3.496	Ethanol, 2-(ethylamino)	$C_4H_{11}NO$	74.33	1.66	
2	4.172	9-Azabicyco[6.1.0]nonane	$C_8H_{15}N$	74.33	0.31	
3	4.37	2-methyl-undecanal	$C_{12}H_{24}O$	81	0.11	
4	4.539	Ethanamine,N,Ndimethyl-[(trimethylsilyl)oxy]	$C_7H_{19}NOSI$	87.66	0.36	
5	4.792	6-Bromohexanoic acid	$C_6H_{11}Bro_2$	96.66	0.14	
6	4.792	N-Methyl-3,4 methylenedioxyamphetamine	$C_{10}H_{13}NO_2$	129	4.45	
7	6.989	(Dimethylamino)ethyl methacrylate	$C_2H_5O_2CC(CH_3)$	177	0.12	
8	7.356	Trans1,4Cyclohexanediame	$C_6H_{14}N_2$	188.33	0.01	
9	7.609	N,N-Dimethyl-4-benzyloxybutylamine	$C_6H_{16}N_2$	195.33	0.01	
10	7.778	5-Hepten-2-amine	$C_7H_{15}N$	201	0.01	
11	10.173	Octadecanoic acid	$C_8H_{16}O_2$	259	3.99	
12	11.018	Thiazol	C_3H_3NS	306.33	4.93	
13	12.454	Propylamine	C_6H_9N	356.33	23.6	
14	13.074	Dodecanoic acid	$C_{12}H_{24}O_2$	389.66	4.15	
15	14.49	Cyclohexanol	$HOCH(CH_2)_5$	403.33	4.92	
16	14.511	1,3-Cyclopentanedione	$(CH_2)_3(CO)_2$	433.33	18.6	
17	14.962	4-methylene- 2-Undecene	$C_{12}H_{24}$	460.66	14.66	
18	15.581	Hydrazine	N_2H_4	469.33	9.83	
19	16.286	Lactone 9-Octadecenoic acid	$C_{18}H_{32}O_2$	504	1.28	
20	16.398	Trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	526	6.87	

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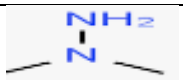
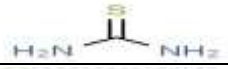
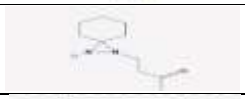
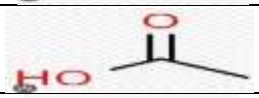
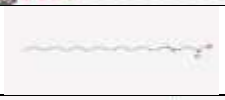
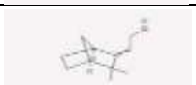
Table 2: Compounds associated with the day 3 fermented seed of *Nigella sativa* characterized by GC-MS

No	Rt (min)	Name of compound	Molecular formula	MW g/mol	Peak area	Structure of the compound
1	2.848	1,1-dimethylhydrazine	$C_2H_8N_2$	62	1.52	
2	3.384	Dimethylethanolamine	$(CH_3)2NCH_2CH_2OH$	111.6	2.04	
3	4.37	2-(Methylamino)ethanol	C_3H_9NO	179.3	0.48	
4	4.539	3-Methylpyridine	$3\text{-}CH_3C_5H_4N$	201.3	0.96	
5	5.102	Acetylcholine bromide	$C_7H_{16}BRNO_2$	252.6	3.9	
6	6.285	N-Methylthiourea	$C_2H_6N_2S$	354.6	0.1	
7	6.482	1,4-diaminobutane	$C_4H_{12}N_2$	366	0.2	
8	8.482	1-(3-Oxobutyl)-3	$C_{10}H_{18}$	485	3.43	
9	9.356	Dodecanoic acid-1-13C	$C_{12}H_{24}O$	596.6	0.29	
10	9.356	Hexanoic acid	$CH_3(CH_2)4COOH$	631.3	1.07	
11	10.651	Tetrahydro-3-methyl-2H-1,3-thiazine	$C_5H_{11}NS$	686	2.27	
12	12.285	2-acetoxymethyl-1,2,3-trimethylbutyl ester	$C_{12}H_{22}O_4$	792	16.11	
13	12.623	3-Heptadecenal	$C_{17}H_{32}O$	873.3	2.17	
14	13.468	2,3-Heptadiene	C_7H_{12}	922	6.97	
15	15.553	6-Octadecenoic acid	$C_{16}H_{32}O$	1,117.60	10.87	
16	16.229	Cyclopropaneoctanoic acid	$C_{11}H_{20}O_2$	1,170.60	1.57	
17	16.37	Cyclopropaneoctanoic acid	$C_{11}H_{20}O_2$	1,191.30	4.66	
18	18.483	6-Octadecenoic acid	$C_{16}H_{34}O_2$	1,355.30	0.17	
19	15.553	6-Octadecenoic acid	$C_{16}H_{34}O_2$	1,117.60	10.87	

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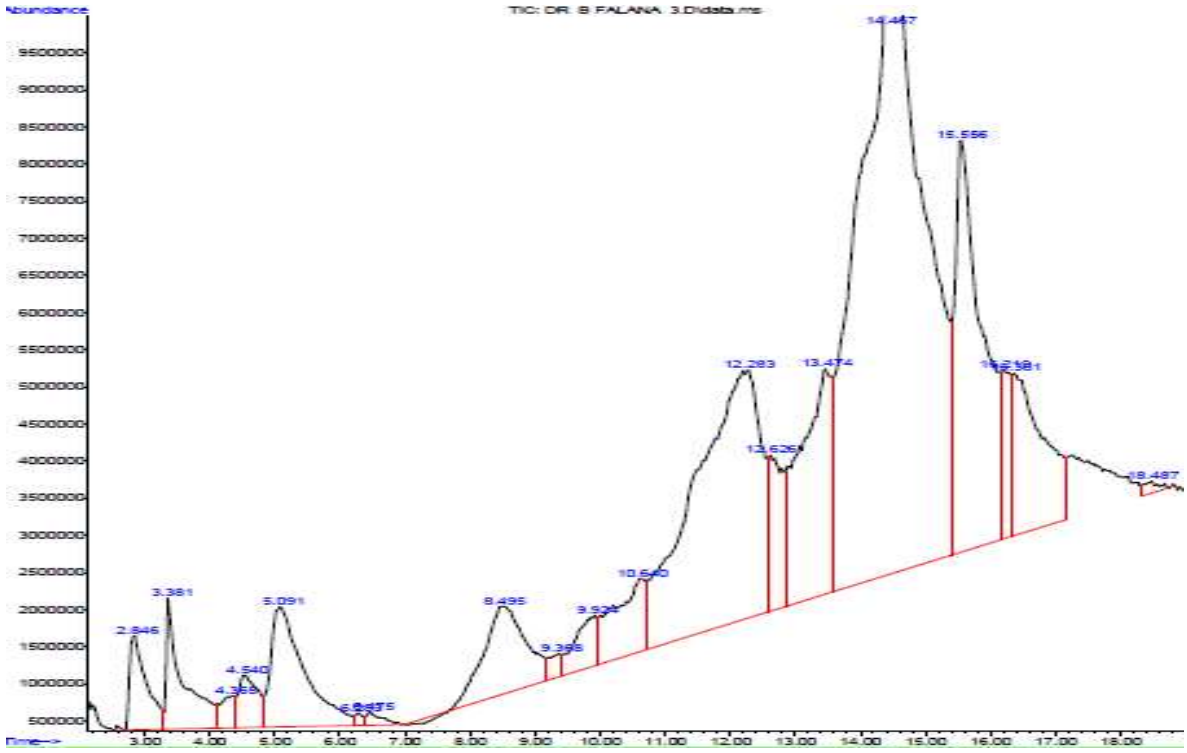

Figure 3: GC-MS chromatogram of the 3-day fermented sample of *Nigella sativa*

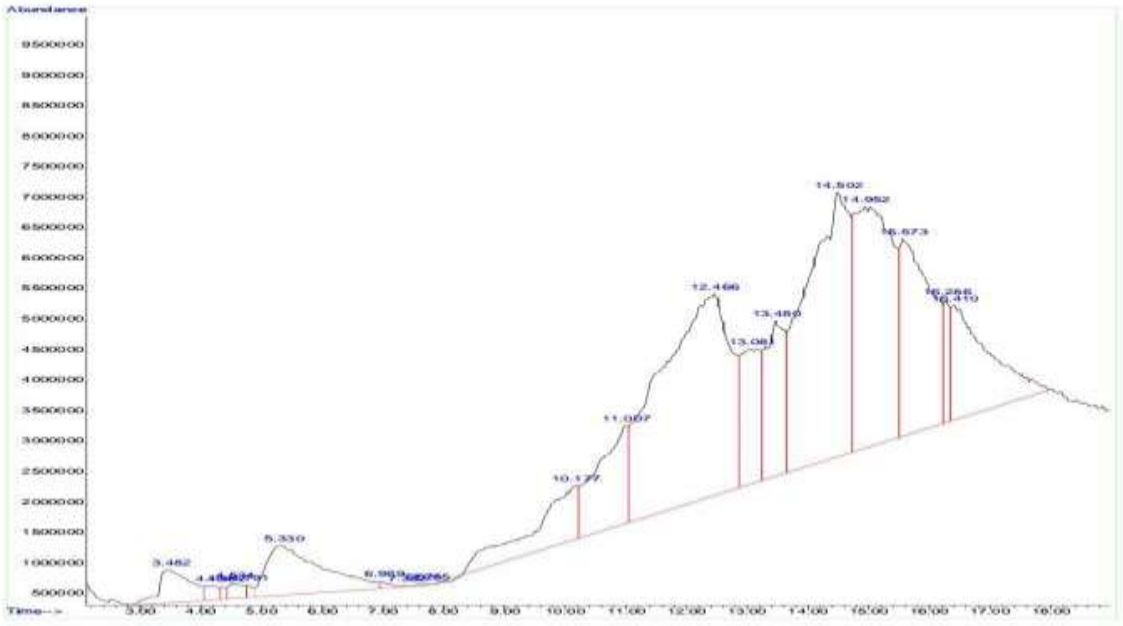

Figure 4: GC-MS chromatogram of the 7-day fermented sample of *Nigella sativa*

The antioxidant capacity of the fermented sample tested at day 3 and day 7 of the fermentation period varied (35.50- 82.69 for the 3-day sample and 37.50-84.99 for the 7-day sample) by the increasing concentration (7.81-1000 mg/mL) of the sample **(Figure 5)**.

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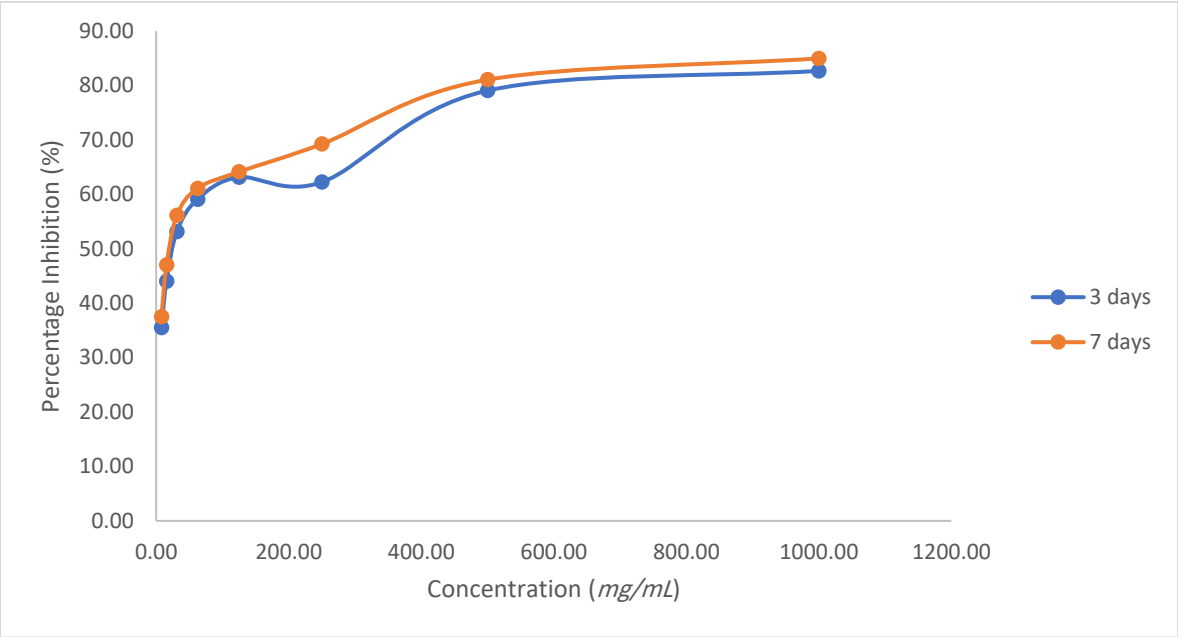

Figure 5: Antioxidant properties of the 3-day and 7-day fermented seeds of *Nigella sativa*

The diameter of zones of inhibition (mm) obtained for the 3-day and 7-day different seeds of *N. sativa* tested at different concentrations (25 mg/mL, 50 mg/mL, and 100 mg/mL) showed varying degrees of activity against *Proteus vulgaris* (**Figure 6**) and *Staphylococcus aureus* (**Figure 7**). The diameter of zones of inhibition varies along the concentration tested with the higher values obtained on the 7-day fermented sample (8-10 mg/mL against *Staphylococcus aureus* and 10-12 mg/mL against *Proteus vulgaris*) than 3-day fermented sample (8-9 mg/mL against *Staphylococcus aureus* and 8-10 mg/mL against *Proteus vulgaris*).

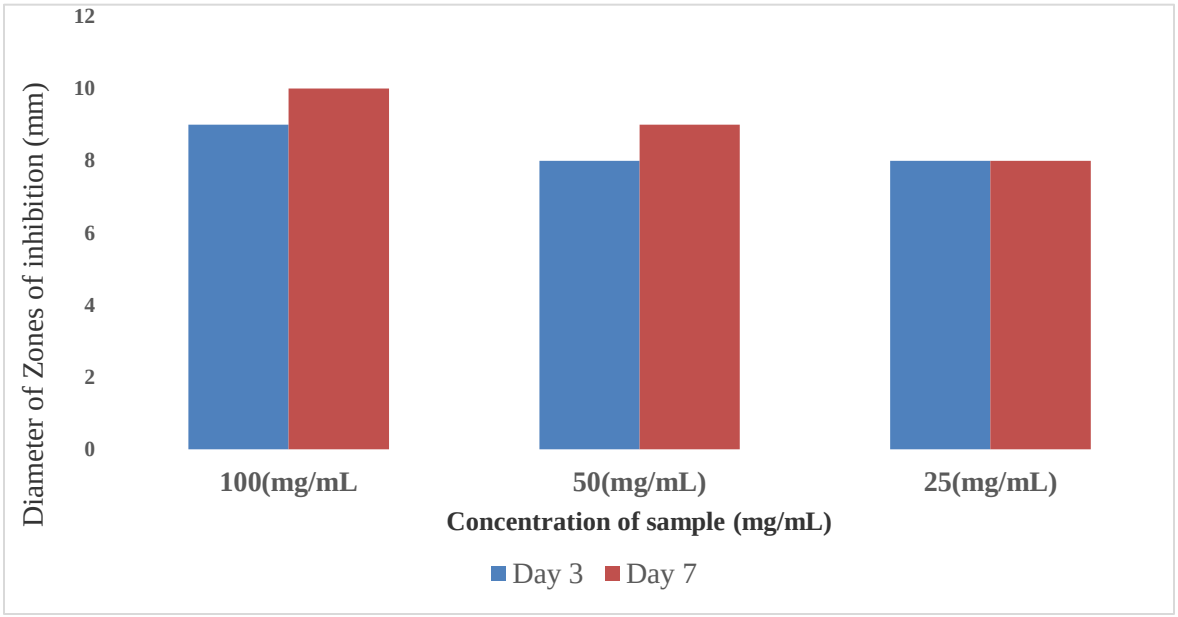

Figure 6: Zones of inhibition of the 7-day fermented seeds of *Nigella Sativa* against *Staphylococcus aureus*

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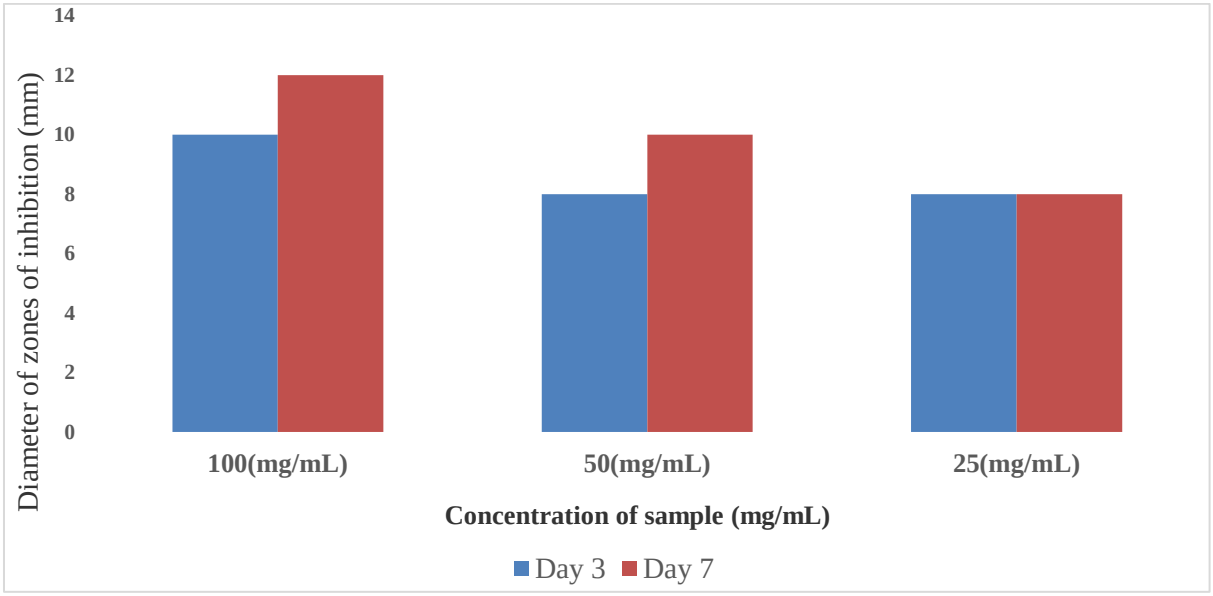

Figure 7: Zones of inhibition of the 7-day fermented seeds of *Nigella sativa* against *Proteus vulgaris*

Discussion

The commonly employed conventional methods to extract bioactive compounds from foods include eco-friendly extraction methods like hydro-distillation, solvent maceration, and Soxhlet extraction [30-32]. However, these techniques are associated with disadvantages such as a long extraction period, selectivity in the extraction of compounds, thermal decomposition of heat-labile compounds, and the use of solvents with high purity, high cost, and potential toxicity [33]. As an alternative, this study employed the process of fermentation, an herbal extraction process that undergoes enzymatic degradation of the plant cell wall which yields better leaching of plant secondary metabolites from the matrix, without the application of high heat, ultrasonic wave, and other radiation sources for extraction which may usually degrade many bioactive constituents, especially phenols.

In this study, a reduction in the pH was observed and this may mean a successful fermentation process signaling an increase in the organic acids produced by associated acid-producing microorganisms [34]. It may also translate to the unsuitability of such an environment for spoilage microbes. Similarly, a decrease in pH was observed in several pieces of scientific reports [35]. Camu and others [36] reported that a decrease in pH observed after day four of fermentation of cocoa beans may be due to the cotyledon pulp sugars being converted into acetic acid through the action of microorganisms such as *Acetobacter rancens* and *Acetobacter melanogenum*. Corroborating the findings of Kabir and associates [37] on the varying nutrition components of *N. sativa*, a similar varying level of the nutritional composition of fermented seeds of *N. sativa* was reported in this study. In the previous study, it was recorded higher protein and fat in *N. sativa* than the current study did, however, the values of carbohydrate, moisture, and ash reported in this study were higher than what was reported on *N. sativa*. This ratifies the statement that fermentation promotes the nutrients in food and eliminates anti-nutrients [38]. The finding in this study is also in line with the report of Hur et al. [39] which stated that the bacterial and endogenous enzymes of grains caused the solubility of some grain constituents leading to the production of new nutritionally active compounds. The high carbohydrates in the fermented samples may be an indicator that the sample contains fermentative organisms that converted major components of the food into useful ones [40].

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The nutritional composition of food significantly contributes to disease prevention and better health promotion. This may account for the phytochemical compounds reported in this study, as phytochemical constituents are commonly regarded as inhibitory substances known to confer antimicrobial properties [41] by inhibiting or slowing the growth of pathogens [42]. Previous scientific findings have reported the fixed oil content of *N. sativa* to be proteins, alkaloids, saponins, and essential oils [43]. In agreement with the report of [44] who stated that flavonoids represent the largest group of phenolics and amount to half of the known phytochemicals in plants, the findings herein also reported flavonoids as the highest phytochemical compounds detected. However, as observed in this study, the decrease in the level of phytochemicals from 3-day fermentation to 7-day fermentation may be attributed to the polymerization by oxidative enzymes leading to the release of phenolics [45]. Maria and others [46] also reported an increase in the total phenolic content during the first 24 hours of fermentation, followed by a slight decrease after 48 hours.

Bioactive phytochemicals in plants often cause adverse anti-nutritional or toxic consequences in other organisms that may be exposed to them and have no direct nutritional value [47]. Fermentation, a biotechnological process, can improve the nutritional value and organoleptic properties of foods by converting conjugated phenols to free forms with the aid of enzymes produced by the associated fermenting microorganisms [13]. This may account for the reduced quantity of phenols recorded in this study. As reported, the level of phenolic compounds in food products could be altered by many factors including methods of processing, such as fermentation [48]. Bioactive compounds (such as gallic acid, quercetin, resveratrol, and epicatechin, among others) have been attributed to the functional characteristics of antioxidant, antimicrobial, anticancer, anti-inflammatory, anti-diabetic, and others [49]. Contrary to the findings in this study where new compounds such as 6-Octadecenoic acid, Cyclopropane-octanoic acid, 3-Heptadecenal, Tetrahydro-3-methyl-2H-1,3-thiazine, hexanoic acid, Acetylcholine bromide, Dodecanoic acid-1-13C, 2,3-Heptadiene, 3-Heptadiene, octadecanoic acid were identified, various scientific reports mentioned Thymoquine as the major compound in *N. sativa* seeds [50, 51]. The newly identified compounds in this study may be due to the biotechnological process of fermentation that has altered the components by the action of enzymes produced by the associated fermenting organisms [13].

In the present study, antioxidant activities of the 3-day and 7-day fermented seeds of *N. sativa* were evaluated at different concentrations and it was found that higher concentration increased antioxidant activity. This corroborates the findings of Ozdemir [52] who reported effective antioxidant activities of *N. sativa* in *in-vivo* and *in-vitro* studies. In addition, the present study demonstrated antimicrobial activity against *Proteus vulgaris and Staphylococcus aureus*. This is in line with the antimicrobial activities of methanolic and ethanolic extracts of *N. sativa* against Gram-positive and Gram-negative bacteria as previously reported by Shahid et al. [53]. Sellami et al. [54] also reported the antimicrobial against Gram-negative. The recorded antimicrobial activities of this study may be attributed to the associated constituents of fermented *N. sativa* seeds earlier reported in this study as it was reported that a variety of secondary metabolites such as tannins, alkaloids, phenolic compounds, and flavonoids, found in plants contribute to their *in vitro* antimicrobial properties [55].

Conclusion: It may be concluded from this study that fermentation is a reliable method of extraction of medicinal components of plants as seen with the seeds of *Nigella sativa*. This study not only shows that fermented seeds of *N. sativa* harbor compounds with proven medicinal potentials but also exhibit some degree of antibacterial activity toward *Proteus vulgaris* and *Staphylococcus aureus*. Thus, fermented seeds of *Nigella sativa* have great potential as an effective antimicrobial agent for medicinal purposes.

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