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Biological and chemical evaluations of the seeds of *Nigella sativa* Linn (Ranunculaceae)

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Abstract
Nigella sativa is a traditional medicinal plant used to manage a number of ailments by many communities in the world, and is native to Asia, Europe, but grown in some parts of Africa, particularly in North East Nigeria where it is used as a dietary supplement. In this study a sample of the seed from North East Nigeria was powdered and extracted with methanol to give the crude extract which was fractionated into hexane, chloroform and methanol solubles. Phytochemical screening of the crude methanol extract showed the presence of the following classes of natural products: alkaloids, tannins, flavonoids, steroids and terpenoids. The proximate analysis of the seed gave 12.00% moisture, 3.00% ash content, 33.30% ether soluble extract, 29.3% crude protein content, 7.2% total carbohydrate, 5.33% crude fibre and 43.30% crude lipids. The seed also had a calorific value of 7203.44 kcal per 100g. The mineral elements determination gave the following composition: Calcium=2.052ppm, Magnesium=0.313ppm, Potassium=498.50ppm, Iron=0.080ppm and Sodium=494.40ppm. The oral *N. sativa* L. seeds treatment decreased the diabetes-induced disturbances of hyper-cholesterolemia, hyperglycaemia and some haematological parameters of alloxan-induced diabetic rats. Given the significant content of fatty acids in the seed, and their known potential applications as antitumor, anti-diabetic and anti-inflammatory agents, the hexane-soluble fraction was subjected to column chromatographic separation to obtain 3 compounds which on spectral characterization led to the identification of octadecadienoic acid, tetradeca-5,9-dien-7,8-dipropionate and tricosane-4,6-dien-12-one. These compounds appear to be new to the plant and may account for some of the traditional medicinal uses of the seed oil, including management of diabetes, hypertension and inflammation.

Keywords: *Nigella sativa* seeds, proximate analysis, oxygenated fats, anti-diabetic activity

Introduction
The use of medicinal plants to treat human diseases has its foundation in pre-historic times. Medicinal plants are used by 80% of the world population as the only available medicines especially in developing countries like Nigeria [1]. Among the promising medicinal plants is black caraway or *Nigella* sativa, a dicotyledonous hardy biennial herb which belongs to the family Ranunculaceae [2]. The plant is indigenous to North Africa, Europe, Western Asia [3] and is also grown widely in Marte local government of Borno State, Nigeria [4]. The ancient Arabs called the seeds "Caraway" or "Habbatul Baraka" and its oil is used as "Habbatus Sawda" while in most cases the black seed is commonly known as "Black cumin" or "Black cara" [5].

Both fruit and oil possesses aromatic and stimulating properties with many folklore uses some of which have been documented by many researchers worldwide [6]. These researchers have reported the ethnomedical and biological activity of the plant. For example, it was widely employed in easing gastrointestinal discomforts and as an immune enhancer [7]. The seeds and oil are also used in a number of medicinal preparations for treating various ailments such as rheumatism, diabetes and hypertension [8]. However, the most significant claim by local traditional practitioners is that the oil cures hypertension. *In vitro* antibacterial effect of the essential oil showed pronounced activity even in 1:1000 dilutions against several organisms that include *Staphylococcus albus, E. coli, Salmonella typhi, Vibrio cholera* [9]. The oil was more effective against gram positive than gram negative organism.

In view of its wide range of medicinal uses, particularly the seed oil, the *Nigella sativa* plant had been under extensive biological and chemical investigations in the past decades. The fixed oil is mainly composed of unsaturated fatty acids that include arachidonic, eicosadienoic and linoleic acid. The saturated fatty acids present in the oil are palmitic, stearic and myristic acids [10]. The essential oil of the seeds was analyzed by gas chromatography-mass spectrometry (GC-MS) and found to contain some quinones [10, 11] as well as monoterpenoids and alkaloids [12] and a triterpene known to have antitumor activity [13]. Also, the ethanolic extract of the seeds was found to contain three flavonoids [14] while the essential oils and their constituents

are known to possess anti-cancer properties [15]. In this work, the results of proximate and elemental analyses, the anti-diabetic activity of the methanol extract in rats and the characterization of three oxygenated long-chain hydrocarbon isolates from the hexane fraction of the seed collected from Maiduguri, Borno State of Nigeria, are reported.

Materials and Methods

Materials

The seeds of *Nigella sativa* Linn were purchased from Maiduguri Monday market in Borno state in November, 2008. The seeds were identified by a taxonomist, Mallam Ibrahim, of the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja. A voucher specimen with NIPRD/H/5160 was deposited at the Institute's Herbarium for reference.

The seeds were thoroughly checked for debris, rinsed with distilled water and air-dried under the shade for two (2) weeks. The dry seeds were ground using a steel-bladed Moulinex coffee grinder to obtain a fine powder and stored in an air-tight cellophene bag until it was required. All solvents used in the work were of analytical grade manufactured by Sigma-Aldrich, but solvents and reagents for spectral analyses were as specified by the equipment manufacturers.

The male rats used for anti-diabetic screening were obtained from the animal house at NIPRD, Idu Abuja.

Each animal had an average weight of 1.5kg and an average age of 12 months. All animals were housed in stainless cages under standard laboratory conditions (light period, 7am-6pm, room temperature, 37 ^{0}C, relative humidity, 45%, ground wheat and soya bean based diet and water *ad libitum*). They also received humane care according to the criteria outlined in the "Guidelines for the Care and Use of laboratory Animals" by the National Academy of Sciences.

All animals were weighed using a digital Metler Balance (Devender Instrument APX-203, Model No: 18009876, Sussex, England) at the end of every week and the handling, management and use of animals for experimentation were as such that allowed minimal stress.

Thin-layer chromatography (TLC) was performed on glass plates (7.5×5.0 cm) coated with Acme's silica gel G containing 13% calsium sulfate as binder. Visualization of spots was done by exposure to iodine vapour and viewing under UV lamp (254+366nm). The isolates were subjected to NMR and MS analyses using Bruker Amx 400 model (100 MHz) and Shimadzu GC-MS QP-5050A, respectively.

Methods

Extraction of seed of *Nigella sativa* with n-hexane

The powdered seed (50g) of *Nigella sativa* was extracted with n-hexane by shaking mechanically with the solvent for 24 hours (200 ml x 3 times). The extracts were pooled together and concentrated under reduced pressure using a rotary evaporator and completely dried *in vacuo*. The extract was then stored in a freezer until required for chromatographic separation

Phytochemical Screening of crude methanol extract

The extract was prepared by macerating about 20g of dried seed powder with 200ml of methanol. The extract was then sunction filtered and the extraction process repeated exhaustively as ascertained by the loss of color of the filtrate. The combined extract was evaporated to dryness *in vacuo* at 45 ^{0}C. The yield of residue was noted and a portion of it was used for the phytochemical screening using standard procedures [16, 17].

Proximate Analysis

The proximate analysis (carbohydrate, fats, protein, moisture and ash) of *N. sativa* seed was determined by using the Association of Official Analytical Chemists (AOAC) [18] methods as outlined below.

The moisture content was determined by drying a known weight of the flour to constant weight in a vacuum oven at 55 ^{0}C. The ash was determined by incinerating the flour in a muffle furnace at 66 ^{0}C till ash was obtained. The crude protein was determined using micro Kjeldahl nitrogen estimation method and multiplying the value for nitrogen by a protein factor of 6.25 to obtain the crude protein. The total carbohydrate was estimated by the Difference Method by subtracting the sum of the values of protein, ash, fibre and lipid from the values of organic matter. The crude lipid was estimated by exhaustive extraction of known weight of dried sample with petroleum ether (bp 40-60 ^{0}C) using a soxhlet apparatus [18].

The crude fibre was obtained from the loss in weight in ignition of dried residue remaining after digestion of fat free sample with 1.25% sulphuric acid 1.25% sodium hydroxide (NaOH) solutions under specified conditions. The caloric value was obtained by multiplying the mean values of the proteins, lipids and carbohydrate by at- water factors of 4, 9 and 4 respectively and taking the sum of the products expressed in kilocalories. The mineral element composition was determined using an atomic absorption spectrophotometer.

Biological Screening Techniques

The following methods were used to determine the physiological effects of the black caraway seeds as a hypoglycemic and hypocholesterolemic agent by determining some haematological values of alloxan-induced diabetic rats.

Preparation of Extract

An extract of the *N. Sativa* L. seeds was prepared using the method described by Meral *et al.*, [19] and Modu, *et al.* [5]. A 5% aqueous extract of the seed was freshly prepared daily by boiling the seeds (50g) in drinking water (100ml) for 10min and then filtering through 4 layers of surgical gauze to obtain the extract.

Anti-diabetic Treatment of Animals

Fifteen male rats, with an average weight of 190 gm and 12 months old, were divided into three experimental groups (control, diabetic and treated-diabetic with *N. sativa* seeds), each group containing 5 rats. At the start of the experiment the animals in groups 2 and 3 were injected intravenously with 150mg/kg of 10% alloxan (Sigma Chemicals co., St. Louis, Mo, USA) dissolved in isotonic sodium chloride to induce diabetes.

The control group was injected only with the same volume of isotonic sodium chloride as the diabetic groups received. Three days after, diabetes mellitus (DM) was confirmed by the demonstration of hyperglycemia (blood glucose, $\geq$300mg/dl). The rats were not treated with insulin at any time during the experiment. The diabetic-*Nigella Sativa* L.-treated group was given the aqueous extract of *N. Sativa* seeds orally at 20ml/g/kg body weight (substitute after the induction of DM was confirmed).

All the animals were housed in stainless cages under standard laboratory conditions and were weighed at the end of every week for two months.

Collection and Hematological Analysis of Samples

The animal treatment experiment lasted for eight weeks, and at the end of this period, the animals in all three groups were fasted for 12 hours and their tail blood samples were collected for determination of fasting glucose levels.

Serum glucose concentration was measured immediately by the glucose oxidase method [20]. The rats were sacrificed 24 hours after the last dose of the extract in the fasting state by decapitation. The liver was immediately extracted, blotted and weighed. Serum was prepared by allowing the blood collected to clot for 1 hour and then centrifuged for 10 mins at 3,000 rpm [21]. An aqueous homogenate was prepared and the supernatant used for the estimation of liver total proteins and the serum cholesterol was estimated by the method of Allain *et al.* [22].

The serum liver total proteins were determined using the method of Teizt [23] while the total lipids were determined according to the method of Chauldhary [21]. Heamoglobin concentration value was determined by the cyonenette-haemoglobin technique [24] while the PCV was measured by the micro heamatocrit centrifuge. Giemsa's stainiry method was used for the differential count of RBC.

Statistical Analysis

The data obtained above were expressed as mean + standard deviation (SD) of five determinations and analyzed using Analysis Of Variance (ANOVA). Tukey's test was used to test for difference values in percentages among means for which ANOVA indicated a significant ($P \leq 0.05$) ratio.

Column Chromatography of the Hexane Extract (Non-Polar Lipids)

Commercially and medicinally, the oil from the *Nigella sativa* seed is of great significance. Consequently, the hexane fraction was investigated for its chemical constituents. Thin-layer chromatography (tlc) of the n-hexane extract showed three prominent spots ($R_{f\ values=}$ 0.70, 0.50 and 0.35; solvent= chloroform: methanol, 95:5) along with some minor spots. The fraction (1.0 g) was applied to a silica gel (100-200 mesh) column and eluted with mixtures of n-hexane and ethyl acetate to yield three components, coded bmm 02, bmm 04 and bmm 07.

Spectral Analysis of isolates

The components, bmm 02, 04 and 07, were subjected to IR, ^{1}H and ^{13}C NMR, and MS spectral analyses.

Resullts and Discussion

The results of phytochemical screening are shown in Table 1.

Table 1 shows the presence of some important classes of compounds with established bioactivities. Tannins are known to reduce the digestibility of nutrients especially proteins, carbohydrates or even lipids by binding to the substrate to be digested and inhibiting digestive enzymes [25].

While saponins are known to be anti-cholesterolemic in nature by reducing cholesterol level in the blood and acting as anti-cancer agent, flavonoids are anti-oxidants and give colour to the plants. Also, alkaloids are anti-glycaemic, anti-bacterial, anti- asthma, anti- cancer and anti-malarial [26].

Table 1: Phytochemical screening of crude methanol extract of *Nigella sativa* seed

Phytochemicals	Remarks
Alkaloids	+
Saponins	+
Tannins	+
Flavonoids	+
Steroids	+
Anthraquinones	+
Cardiac glycosides	+
Phlobatannins	+
Cardenolides	+
Terpenoids	+
Key: (+)=Present	

The elemental conposition of *Nigella sativa* seeds (Table 2) shows it is a rich source of potassium, sodium and phosphorus, with values higher than those reported for some other seeds such as *Bolanites Aegyptieca* and *Tamarindus indica* [27]. These minerals are usually required in small amounts in the body and their main function is to act as essential co-factors in enzymatic reactions.

The *Nigella sativa* oil physicochemical characteristics are presented in Table 3. The iodine, acid and peroxide values of oil influence their oxidative stability. The iodine values of 117 and 115 in hexane and ethylacetate, respectively, are lower than 122.3 recorded for *C. vulgaris* [28]**.** The iodine value is an index of unsaturation in a fatty acid. The acid value represents free fatty acid content due to enzymatic activity in the seeds. High acid values are usually indicative of damage or high seed moisture content which enables the enzyme lipase to convert triglycerides to free fatty acid. The maximum acceptable value is 4 mg $KOHg^{-1}$ [28]. The saponification value of 200 mg $KOHg^{-1}$ indicates a preponderance of fatty acids with low molecular weights of 188.13 and 184.14 in hexane and ethyl acetate, respectively. The saponification value obtained for this seed extract shows the oil to be made of medium and long-chain fatty acids.

Table 2: The results of determination of elemental composition of *Nigella sativa*

Element	Concentration(mg/100g)
Calcium	2.05±1.20
Potassium	498.50±1.10
Magnesium	0.313±0.05
Iron	0.488±080
Sodium	494.40±1.40
Phosphorus	50.30±1.70

The ash content of the seed was found to be 3.00%. The determination of ash content is of value in the analysis of food for vital reasons and is used as an index of the quality of the seed as feedstock for poultry, men, and cattle [29]. The seeds contain about 43.30% of crude lipid and this is in line with the fact that it is an oil seed.

Lipids have three important functions in foods; culinary, physiological and nutritional. The dietary lipids provide linoleic acid which stabilizes the cell membranes and prostaglandin which has the effect of inhibiting inflammation.

Table 3: Physicochemical Properties of *Nigella sativa oil*

Parameters	Hexane	Ethyl Acetate
Colour	Deep amber/ dark	Deep amber/ dark
Iodine value (mgi$_2$/100g)	117.00	115.00
Saponification (mgKOH)	188.13	184.14
Acid value (mgKOH)	0.60	0.55
Peroxide value (mEq/kq)	0.40	0.45
Degree of unsaturation (%)	80.00	80.00
% Impurities	35.00	30.00
PH value	4.16	4.16
% Free fatty acids	0.846	0.776
Octane number	35.00	34.00
Mono unsaturated FAME (%) 80.00		80.00

Table 4: Results of proximate analysis of *Nigella sativa* seeds (dry weight basis ± standard deviation)

Parameter	Composition
% moisture	12.00 ± 0.03
% crude lipid	43.30 ± 1.10
% crude protein	29.30 ± 0.08
% crude fiber	5.33 ± 1.02
% Carbohydrate	6.20 ± 0.12
% Organic matter	33.3 ± 0.03
%Ash	3.00 ± 0.01
% Caloric value	7203.44Kcal/100g

In Table 4 the crude proteins and total carbohydrate contents (29.30±0.08% and 6.20± 0.12%, respectively) were high compared to those reported for some less important plant seeds [30]. For example the crude protein content of coconut water, chestnut and dried corns range from 3.50 to 8.10%. Also, seeds of *Solanum nigrum var virginicum* have protein content of 17.63% [31].

The crude fibre content was found to be 5.33±1.02% which is quite high, thus accounting for the potential of the seed or its oil in the management of diverticulitis, cancer of the colon, cardiovascular disease and diabetes mellitus [32, 33]. The importance of high fibre grains in the control of blood sugar and hypertension have been well known [34].

The anti-diabetic and anticholesterol properties of the seed are thus strongly supported by the results of anti-diabetic screening presented in Table 5, with the extract treated diabetic animals showing better health indices than the untreated diabetic and comparable to the control.

Table 5: Comparative heamatological values, serum glucose concentration, serum, liver lipids, cholesterol and proteins

Parameters	Control group	Diabetic group	Diabetic with *Nigella sativa* treatment
1. RBC (X106/NL)	6.5±0.3a	5.2±0.2b	6.0±0.7a
2. Hb (G/dl)	11±0.4	12±0.1	11±0.6
3. PCV %	20±0.5a	14±0.3b	18±0.4a
4. Glucose mg/dl	80.3±2.0a	240.2±15b	170.0.3±10.00c
5. Serum cholesterol mg/dl	12.34±0.12a	17.30±0.30c	11.92±0.22
6. Serum total lipids mg/dl	15.11±0.11	15.48±0.24	16.11±0.01
7. Serum total protein	17.01±0.2a	22.01±0.2b	17.62±0.3a
8. Liver total lipids	68±5.0a	79.1±0.2b	48.2±3.0c
9. Weight in grams	190	170	180

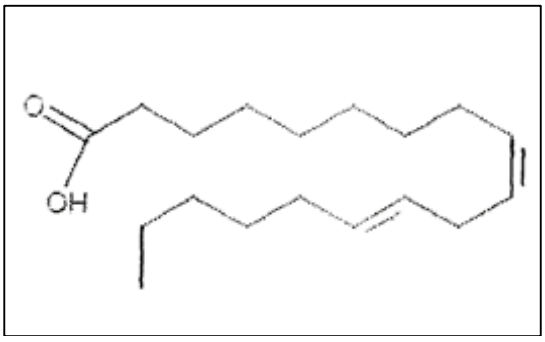
- Values represent the means + SD (n=5) abc: means in the score now with different superscripts significantly differ from each after P<0.05. Number of rats in each group = 5

Chromatographic purification of the hexane-soluble fraction of Nigella seed gave 3 compounds which were subjected to spectroscopic analysis.

The IR spectrum (cm^{-1}) of compound bmm 02 showed absorptions at 3416.28(OH) for carboxylic acid hydroxyl group, 2762.53- 3009.37(C-H, broad) for hydrocarbon, 1737.55(C=O) for acid carbonyl function. The ^{1}H NMR ($CDCl_3$) showed signals at 10.3(1H, s) for acid hydroxyl, 5.27-5.39(4H, m) for ethylenic protons, 2.74-2.75(2H, m), 2.27-2.3 (2H, m), 2.00-2.07(4H, m), 1.60-1.67(2H,), 1.26-1.40 [-CH_2]n-, complex, 0.87-0.94 (CH3, t). The ^{13}C NMR showed a peak at 180.3 for carboxylic acid carbonyl carbon and signals at 127.9, 128.1, 129.9, 130.1 for four ethylenic carbons while the DEPT-135 spectrum showed peaks above at 14.1 for methyl and 127.9, 128.1, 129.9, 130.1 for four ethylenic carbons, while the peaks at 22.7, 24.6, 27.2, 29.1, 29.3, 29.7, 31.5 and 34.1 for methylene carbons occurred below. The EIMS (m/z) gave a molecular ion peak at m/z 280. The above spectral characteristics are suggestive of octadecadienoic acid, 1 [35] and this has been further supported by direct comparison with computer Library mass spectral data.

Compound bmm-04 which was obtained as oil showed the following spectral characteristics: IR Spectrum (cm^{-1}): 2854.13, 2924.52 and 3008.41 for C-H stretching of hydrocarbon chain, 1746.23 for saturated ester carbonyl function and 1659.45 for C=C function. The ^{1}H NMR ($CDCI_3$+CCl_4) spectrum gave resonance signals at 5.27-5.39 (4H, m) for ethylenic protons, 4.26-4.30(1H,m) and 4.09-4.13(1H, m) for two separate oxymethine protons and 2.74-2.75 (2H, m), 2.27-2.3 (2H, m) , 2.00-2.07(4H, m), 1.60-1.67(2H, m), 1.26-1.40[-(CH_2)n-, complex], 0.87-0.94(CH_3, t).These are suggestive of the presence of a long hydrocarbon chain. The ^{13}C NMR spectrum ($CDC1_3$+CCl_4) spectrum showed signals at 14.13, 14.16 for methyl carbons, 22.7, 24.6, 27. 19,27. 2,29.1,29.2,29.3,29.7, 31.5 and 34.1 for methylene carbons, 62.00 and 68.87 for two oxymethine carbons and supporting the presence of two ester groups, 127.9, 128.1, 129. 9,130.1 for four ethylenic carbons, and 172.4 and 172.8 for two ester carbonyl carbons. The DEPT-135(NMR)

showed peaks above at 14.1 for methyl and 127.9, 128.1, 129. 9,130.1 for four ethylenic carbons, while peaks at 22.7, 24.6, 27.2, 29.1, 29.3. 29.7 31.5 and 34.1 for methylene carbons occurred below. In the absence of a methoxy signal in the ^{1}H and t ^{13}C NMR spectra the two ester groups are probably oxymethine which are reflected by the peaks in the DEPT-135 spectrum at 68.87 and 61.9 for two carbons in slightly different spatial orientations. The EIMS (m/z) showed a molecular ion at m/z 339 [M+l] with a base peak at m/z 55. Thus, bmm-04 may be the unusual and probably new diester, tetradecane-5,9-dien-7,8-dipropionate (2).



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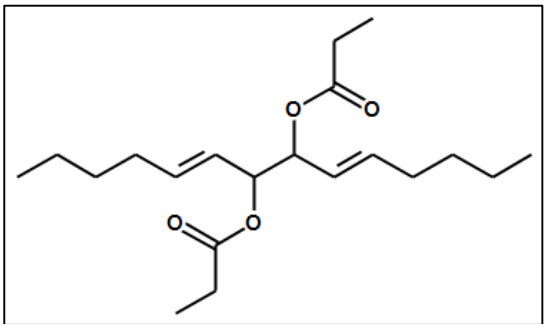
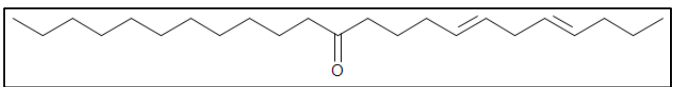


2

The IR spectrum of compound bmm 07 did not exhibit any OH absorption, but displayed C=O overtone at 3645, a strong carbonyl absorption at 1713 suggestive of a saturated ketone. The DEPT spectrum showed two sets of four ethylenic carbon peaks similar to compounds bmm 04, suggesting that that it has two ethylenic bonds in the molecule. In addition the HRMS (m/z) showed a molecular ion peak at 334.3236. On the basis of the above spectral characteristics and by comparison with those recorded for compounds bmm 02 and 04, the compound bmm07 has been tentatively assigned structure 3, representing tricosane-4,6-dien-12-one (a C_{23} aliphatic compound). n-Tricosane and 9-tricosene have previously been identified as volatile organic components of the flowers, stem and roots extracts of *Tripleurospermum callosum* using GC-MS analysis [36]. They are also known to be components of the female sex pheromone of the bee, *A. nigroaenea*, while a related compound, 6,9-tricosa-diene is a component of the female produced pheromone of the wasp, *Euryptoma amygali* [37].



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Conclusion

The phytochemical screening of *Nigella sativa* seed methanol extract showed the presence of some important classes of natural products such as alkaloids, flavonoids, tannins, saponins and terpenoids, among others.

Elemental, physicochemical and proximate analyses revealed that the seed contains some essential minerals, fatty acids and good nutrients for the maintenance of good health. In particular the seed has very high fibre content which accounts for its potential to control diabetes mellitus and hypertension. A number of Covid-19 patients have recently attested to the potency of the oil in boosting immunity and enhancing their recovery.

The results obtained from the spectral analysis and GC-MS had shown the seed oil from *Nigella* sativa to contain three oxygenated long-chain fats, including 9,12,-octadecadienoic acid, tetradecane-5,9-dien-7,8-diproprionate and tricosane-4.6-dien-12-one. These long-chain aliphatic oxygenated compounds appear to be new and add to the list of compounds previously reported from *Nigella sativa* Linn.

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