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Original Article

# Biological, phytochemical, and physico-chemical properties of two commercial *Nigella sativa* seed oils: A comparative analysis

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**ABSTRACT**

This study was designed to evaluate and correlate the biological, phytochemical, and physicochemical properties of two NSS (*Nigella sativa* seed) oils (N1 and N2). The biological activity was evaluated in terms of antibacterial, antioxidant (nitric oxide scavenging), antielastase, antityrosinase, antimelanogenic, and anticancer activity. Physicochemical properties including colour, total soluble solids (TSS), and density were also investigated. N2 exhibited higher antibacterial, NO scavenging, extracellular antimelanogenic, and anticancer activity against the HeLa cell line compared to N1, which showed higher antityrosinase activity. TPC (total phenolic content) and TFC (total flavonoid content) of N1 were significantly higher than N2 while TTC (total tannin content) was higher in N2. In fact, TTC was strongly correlated (R=1.000) with the antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity. To conclude, NSS oil may be considered as a complementary and alternative therapy in the management of infectious and chronic diseases but warrants further *in vivo* pharmacological validation and toxicological analysis.

**Keywords:** *Nigella sativa*, biological, phytochemical, physicochemical

## INTRODUCTION

The growing number of bacterial infections and antibiotic resistance have become a major threat to global health, food security, and development. Without effective antibiotics, modern medical treatments including organ transplantations, chemotherapy, and surgeries become much more risky (WHO 2017a). On the other hand, noncommunicable diseases (NCDs) are now the leading cause of death globally, responsible for 40 million deaths each year, equivalent to 70% of all deaths across the world (WHO 2017b). Many NCDs are associated with an increased oxidative stress which is caused by an imbalance between excess free-radical production and endogenous antioxidant levels in the body (Pham-Huy et al 2008). Among NCDs, cancer is the second leading cause of death, accounting for 8.8 million deaths in 2015 (WHO 2017c).

Besides the exploration of antioxidants and chemotherapeutic drugs in the management of NCDs such as cancer, research on the inhibition of key enzymes in the body for the treatment of NCDs has recently intensified. For instance, inhibition of tyrosinase, a key enzyme involved in melanin biosynthesis, may prevent excess formation and accumulation of melanin in the skin, preventing hyperpigmentation disorders including melasma, freckles, lentigines, and geriatric pigment spots (Ya

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et al 2015; Aumeeruddy et al 2017). In addition, over-enzymatic activity, particularly elastase, is associated with loss of skin elasticity; one of the classical aging characteristics. Elastase breaks down elastin, a constituent of the connective tissue responsible for skin firmness and elasticity (Mathen et al 2014).

Recently, much attention has been focused on the exploration of natural remedies for therapeutic purposes due to their low cost, and because of the association of side effects to synthetic drugs. Among natural resources such as terrestrial and marine plants, animals, and micro-organisms, plants have always been a versatile source of shelter, clothing, food, flavours and fragrances, and not the least, medicines (Gurib-Fakim 2006). In fact, 11% of the 252 drugs, which are considered as basic and essential by the World Health Organisation, are exclusively of plant origin or synthetic drugs derived from natural precursors (Rates 2001). In addition, phytochemistry has become a field of active interest for drug discovery and formulations through isolation, purification and characterisation of new phytochemicals, the biologically active compounds found in different parts of plants ( Ahmad et al 2013).

*Nigella sativa* seeds (NSS), also known as black seed (English), çörek otu (Turkish) habat-ul-sauda (Arabic) and kalonji in South Asia, is the black coloured, funnel shaped seeds of the *N. sativa* plant which belongs to the Ranunculaceae family. The plant is cultivated in various regions such as Southern Europe, North Africa, Middle Eastern Mediterranean and the southern regions of Asia including Syria, Turkey, India, Pakistan, and Saudi Arabia (Gilani et al 2004). NSS is regarded as a valuable traditional remedy and has been found to possess extensive biological properties, including antimicrobial, antioxidant, antiinflammatory, anticancer, antidiabetic, cardioprotective properties amongst others (Ahmad et al., 2013). Nonetheless, due to variations observed among studies because of geographical origin, climatic conditions, variety, agricultural techniques applied, extraction and processing techniques, and storage conditions, further analysis is required to understand the factors responsible for these variations in order to obtain better medicinal products and enhanced therapeutic efficacy. The present study therefore aimed to determine any differences in biological activity of two NSS oils originating from the same country, and correlate the data obtained in relation to their phytochemical composition and physicochemical properties. Biological activity was assessed in terms of antibacterial, antioxidant, antielastase, antityrosinase, antimelanogenic, and anticancer activity.

## MATERIALS AND METHODS

### Materials

Commercially available cold-pressed (expelled) NSS oil (N1) and NSS oil (N2), unspecified regarding its extraction technique, were obtained from shops in Mauritius. The NSS oil samples were stored at room temperature in the dark for the entire duration of the study.

### Reagents

All chemicals and reagents used in the study were of analytical grade and were purchased from reliable firms and institutes. Porcine pancreatic elastase type IV, N-succinyl-(Ala)3-p-nitroanilide, Trizma base, XTT cell proliferation kit II, L-ascorbic acid, and Actinomycin D were obtained from Sigma Aldrich, MO, USA. Mushroom tyrosinase, L-tyrosine and koijc acid were obtained from Sigma Aldrich, Johannesburg, RSA. The human cervical adenocarcinoma (HeLa) and human breast adenocarcinoma (MCF-7) cell lines were obtained from the European Collection of Cell Cultures (ECACC, England, UK). Minimum Essential Medium (MEM), trypsin-EDTA, fetal bovine serum (FBS), phosphate buffer saline (PBS), Mueller-Hinton agar (MHA), Mueller-Hinton broth (MHB), and antibiotics were supplied by Thermofisher scientific (Modderfontein, Johannesburg, RSA). Sodium nitroprusside and Griess-Ilosvsy's nitrite reagent were purchased from Merck Millipore, Darmstadt, Germany.

### Antibacterial assays

Disc and well diffusion methods were performed following the guidelines of "The Clinical and Laboratory Standards Institute (CLSI)" (CLSI 2015). The two assays were carried out in parallel and parameters including inoculum level, depth of agar, and size of disc and well, were kept constant. Measurements were carried out in triplicate.

### Microorganisms used for antibacterial assay

Clinical isolates including *Proteus* sp., *Klebsiella* sp., *Streptococcus* sp., *Pseudomonas* sp., and *Escherichia coli* were obtained from the Faculty of Science, University of Mauritius, while American Type Culture Collection (ATCC) strains including *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis* ATCC 35984, and *Staphylococcus epidermidis* ATCC 14990 were obtained from the Faculty of Agriculture, University of Mauritius. All strains were sub-cultured on Mueller-Hinton Agar (MHA) and grown in Mueller-Hinton broth (MHB) at 37 °C prior to the day of use.

### Disc Diffusion Assay

One hundred microlitres of bacterial culture, adjusted to 0.5 McFarland standard turbidity scale in MHB, was spread evenly on the surface of MHA plates. Paper discs (5 mm), prepared from Whatmann filter paper, were impregnated with 30 µl of NSS oil (undiluted), and placed on the inoculated plates. Discs impregnated with 30 µl of streptomycin, cloxacillin, ampicillin, and chloramphenicol, at concentration 1 mg/ml, were used as positive controls, while sterile distilled water was used as the negative control. Plates were incubated at 37 °C for 24 h and the diameter of zone of inhibition (ZOI) including that of the discs were measured in mm.

### Well Diffusion Assay

One hundred microlitres of bacterial culture, adjusted to 0.5 McFarland standard turbidity scale in sterile MHB, was spread

evenly on the surface of MHA plates. Five millimeter diameter wells, sufficiently spaced to avoid overlapping of results, were punched into the surface of the agar using a sterile cork borer followed by addition of 30 µl of NSS oil (undiluted) to each well. Four antibiotics were used as positive control including streptomycin, cloxacillin, ampicillin, and chloramphenicol, at a concentration of 1 mg/mL, while sterile distilled water was used as the negative control. Plates were incubated at 37°C for 24 h and the diameter of the ZOI including that of the well were measured in mm.

**Antioxidant assay**

**Nitric oxide scavenging assay**

The nitric oxide (NO) scavenging activity of the samples was measured according to the method described by Mayur et al (2010). The oil samples were prepared by dissolving the oil in ethanol to a starting concentration of 10 mg/mL. To the top row of a 96-well plate, 20 µL of distilled water and 80 µl of the NSS oil sample were added. The NSS oil samples were double diluted to a final concentration ranging from 2000 µg/mL to 15.6 µg/mL. Ten millimolar sodium nitroprusside (50 µL) was added to all the wells followed by incubation at room temperature under light for 90 min. After incubation, Griess-Ilosvsy's nitrite reagent (100 µL) was added to the test wells and distilled water to the colour control wells. The nitrite content was measured at 546 nm after 5 min incubation in the dark. L-ascorbic acid (10 mg/mL) and ethanol was used as the positive and negative controls, respectively. The radical scavenging activity was determined as percentage NO radical-scavenging activity which was calculated by the equation: NO radical-scavenging=[(AC-AS)/AC]×100; where AC is the absorbance of the control solution that contains only NO, and AS is the absorbance of NSS oil samples in NO solution. From these results, the fifty percent inhibitory concentration ($IC_{50}$) was determined using the GraphPad Prism 4.0 program (GraphPad Software, Inc., CA, USA).

**Elastase inhibitory activity**

The ability of the NSS oil samples to inhibit porcine pancreatic elastase (PPE) was determined by measuring the release of *p*-nitroaniline from N-succinyl-(Ala)3-p-nitroanilide spectrophotometrically according to the method of Bieth et al (1974) with slight modifications. The reaction mixture contained 100 mM Tris buffer (pH 8.0), 0.5 M HCl, and the test sample (NSS oil and the positive drug control, ursolic acid) which were serially diluted to yield a concentration range of 250-3.13 µg/ml. PPE (5 mM) was then added and the reaction mixture was incubated for 15 min followed by the addition of N-succinyl-(Ala)3-p-nitroanilide (4 mM). A vehicle control where the sample was replaced by methanol was included as the 100% rate, and 0% where the enzyme and substrate were replaced, respectively, by buffer solution. The change in the absorbance of the reaction mixture was measured kinetically at 405 nm for 15 min using KC Junior software and a BIO-TEK Power-Wave XS multiwell plate reader. One unit of elastolytic activity is defined as the release of 1 µM of p-nitroaniline/min. The concentration of NSS oil at which fifty percent of the enzyme was inhibited ($IC_{50}$) was then calculated.

**Tyrosinase inhibitory activity**

The antityrosinase assay was performed according to the method described by Mapunya et al (2011), with few modifications. The NSS oil samples were dissolved in 100 µl DMSO to a 20 mg/ml stock solution which was diluted with 50 mM potassium phosphate buffer (pH 6.5). In a 96-well microtitre plate placed on ice, 30 µl of tyrosinase enzyme (333 units/ml in phosphate buffer pH 6.5) was added to 70 µl of varying concentrations of NSS oil, in triplicate. After 5 min of incubation on ice, 110 µl of substrate (2 mM L-tyrosine) was added to all the wells. The final concentrations of the sample and positive control (kojic acid) ranged from 1000 to 1.5 µg/ml. The optical density (OD) was then measured over a period of 30 min at a wavelength of 492 nm using BIO-TEK power Wave XS multi-well plate reader (KC Junior). The fifty percent inhibitory concentration ($IC_{50}$) was then determined by analysing the resulting data using the software GraphPad Prism 4.0 (GraphPad Softwar, Inc., CA, USA).

**Melanin inhibitory activity**

**B16F10 Melanoma Cell culture**

Mouse melanocytes (B16F10) were cultured in complete Minimum Essential Eagle's Medium (MEM), containing 10% FBS, 1.5 g/L $NaHCO_3$, 2 mM L-glutamate, 10 mg/ml streptomycin, and 0.25 mg/ml fungizone.

**Measurement of melanin production in Cultured B16F10 Melanoma Cells**

The inhibitory effect of NSS oil on melanin production was determined following the Hill method previously described by Matsuda et al (2005). The cultured B16F10 mouse melanoma cells were trypsinised (0.25% trypsin and 0.1% EDTA at 37 °C for 5-10 min) and plated into 24-well plates ($5 \times 10^4$ cells/well in 1.5 ml of MEM). The plated cells were incubated for 24 h at 37 °C in the $CO_2$ incubator. Following incubation, 500 µl of each NSS oil sample (concentration ranging from 500 to 15.6 µg/ml) was added to each well in duplicate, and the treated 24-well plates were incubated for 3 days at 37 °C in the $CO_2$ incubator. Test samples and theophylline (negative control) were dissolved in DMSO. The final concentration of DMSO was 5%. The untreated cells were used as the control group.

After incubation, the cultured medium was removed by a pipette and assayed for extracellular melanin as follows: The cultured medium was centrifuged (900 g, 20 min at 4 °C) to separate the cellular components and extracellular components. One millilitre of a mixture of 0.4 M Tris buffer (pH 6.8) and ethanol (9:1, v/v) was added to 1 ml of the supernatant. The OD of the resulting solution was then measured at 475

nm, and the amount of extracellular melanin was determined.

To determine the intracellular melanin production, the remaining melanoma cells were washed with CMF-D-PBS (Calcium and Magnesium Free Dulbecco's-Phosphate Buffered Saline) and trypsinised (100 µl of 0.25% trypsin and 0.1% EDTA at 37 °C for 5-10 min). The cells were digested by the addition of 400 µl of 1 N NaOH and then left standing for 16 h at room temperature. The OD of the resulting solution was then measured at 475 nm, and the amount of intracellular melanin was determined. Melanin inhibition was determined by comparing the OD of the dose dependent treated cells with the untreated cells and the $IC_{50}$ values were determined.

**Anticancer activity**

**Cell culture**

The human breast adenocarcinoma (MCF-7) and human cervical adenocarcinoma (HeLa) cell lines were maintained in MEM supplemented with 10% FBS, 1% antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin), and 250 µg/ml fungizone. The cells were grown statically at 37 °C in a humidified incubator set at 5 % $CO_2$. Once confluent, the cells were sub-cultured by treating them with trypsin-EDTA (0.25 % trypsin containing 0.53 mM EDTA) for a maximum of 15 min.

**MCF-7 and HeLa cell inhibition**

The cytotoxicity of NSS oil was evaluated using the XTT cell proliferation Kit II according to the method of Zheng et al (2001). MCF-7 and HeLa cells (1 x $10^5$ cells/ml) were seeded in 96-well microtiter plates, respectively, and allowed to attach for 24 h at 37 °C and 5 % $CO_2$. The NSS oil samples were prepared at 20 mg/ml stock concentrations in DMSO. The cells were treated with NSS oil at concentrations ranging from 400-3.13 µg/ml and the positive drug control, actinomycin D, with concentrations ranging between 0.5 µg/ml and 0.002 µg/ml. A vehicle control was included where cells were treated with 2 % DMSO. The treated cells were incubated for 72 h followed by the addition of 50 µl XTT to a final concentration of 0.3 mg/ml. The plates were incubated with the viability reagent for 2 h and the absorbance of the colour complex was measured at 490 nm with a reference wavelength set at 690 nm for XTT using KC Junior software and a BIO-TEK Power-Wave XS multi-well plate reader. The assay was performed in triplicate and the fifty percent inhibitory concentration ($IC_{50}$) values of the samples were calculated using the GraphPad Prism 4.0 program (GraphPad Software, Inc., CA, USA).

**Phytochemical analysis**

**Qualitative phytochemical test**

Phytochemical screening for the presence of alkaloids, flavonoids, saponins, phenols, anthraquinones, and steroids was performed using standard protocols (Andzouana and Mombouli 2011; Tiwari et al 2011).

**Quantitative phytochemical test**

Total phenolic content (TPC) was measured using the Folin–Ciocalteu method as described by Picot et al (2014). Five hundred microlitres of test sample was mixed with 2500 µl Folin–Ciocalteu reagent (ten-fold diluted) and 2000 µl of sodium carbonate solution (7.5 %). The mixture was allowed to stand for 30 min and the absorbance of the solution was measured spectrophotometrically at 760 nm. All determinations were performed in triplicate. TPC was expressed as microgram of gallic acid equivalent (GAE) per gram of sample (µg GAE/g sample).

The total flavonoid content (TFC) was determined using the aluminum chloride colorimetric method previously described by Picot et al (2014) . The reaction mixture containing 2 ml of diluted sample and 2 ml of 2 % $AlCl_3$ solution was allowed to stand at room temperature for 30 min after which the absorbance of the solution was measured spectrophotometrically at 420 nm. All determinations were performed in triplicate and TFC was expressed as µg of rutin equivalent (RE) per g of sample (µg RE/g sample).

Total tannin content (TTC) was determined using the vanillin-HCl method as previously described by Mak et al (2013). 1 ml of sample was mixed with 5 ml of the reagent mixture (4 % vanillin in methanol and 8 % HCl in methanol in the ratio of 1:1). After 20 min, the resulting color change was measured spectrophotometrically at 500 nm. TTC was expressed as µg catechin equivalent (CE) per g sample (µg CE/g sample).

**Physicochemical properties**

Physicochemical properties including colour, TSS, and density were tested. For colour measurement, CIELAB L* a* b* colour parameters were determined using a chromameter (Minolta CR-410, Konica Minolta, Japan), which was placed directly over the samples in petri dishes filled to the brim. L* represents lightness, a* measures the degree of red (+a*) or green (-a*) colours and b* parameter indicates the degree of the yellow (+b*) or blue (-b*) colours (Boussaid et al 2014). In addition, TSS was measured using a digital hand-held "Pocket" refractometer (ATAGO, PAL-3) with ranges of 0-93˚Brix. Density was measured according to the method described by Kinoo et al (2012) using the formula: Density = Mass of sample/volume of sample. All measurements were done in triplicate.

**Statistical analysis**

All data presented in this study was analysed using Microsoft Excel 2010, Minitab version 16, and GraphPad Prism 4.0. One way ANOVA (Tukey's test) was used for evaluation of significant differences between the variables. Pearson's correlation was used to evaluate correlation between the variables. $P<0.05$ was considered statistically significant.

**RESULTS AND DISCUSSION**

**Antibacterial activity**

The results of the antibacterial activity of NSS oil are shown in Table 1 and Table 2. The bacterial strains displayed variation

**Table 1. Antibacterial activity of undiluted samples using disc diffusion assay**

| | N1 | N2 | Streptomycin | Ampicillin | Cloxacillin | Chloramphenicol |
|---|---|---|---|---|---|---|
| *E.coli* (clinical isolate) | 7.3±0.58$^{c}$ | 8.0±0.0$^{c}$ | 10.7±0.58$^{b}$ | NI | NI | 21.0±1.0$^{a}$ |
| *E.coli* ATCC 25922 | 10.0±1.0$^{c}$ | 10.3±0.58$^{c}$ | 25.3±0.58$^{a}$ | 13.0±0.0$^{b}$ | NI | 25.7±0.58$^{a}$ |
| *Proteus* sp. (clinical isolate) | 8.7±0.58$^{c}$ | 9.0±0.0$^{c}$ | 18.7±0.58$^{a}$ | NI | NI | 15.7±0.58$^{b}$ |
| *P. mirabilis* ATCC 12453 | 33.0 ±1.0$^{b}$ | ≥ 44$^{a}$ | 23.7±0.58$^{e}$ | 20.0±1.0$^{f}$ | 28.7±0.58$^{c}$ | 26.7±0.58$^{d}$ |
| *Pseudomonas* sp. (clinical isolate) | 7.7 ±0.58$^{a}$ | 8.0±1.0$^{a}$ | NI | NI | NI | NI |
| *P. aeruginosa* ATCC 27853 | 15.3±0.58$^{d}$ | 31±1.0$^{a}$ | 25.7±0.58$^{b}$ | 15.3±0.58$^{d}$ | 13.7±0.58$^{e}$ | 23.0±0.0$^{c}$ |
| *Klebsiella* sp. (clinical isolate) | 8.0±1.0$^{b}$ | 8.7 ± 0.58$^{b}$ | 16.3±0.58$^{a}$ | NI | NI | 8.0±0.0$^{b}$ |
| *Streptococcus* sp. (clinical isolate) | 19.7±0.58$^{b}$ | 39.7 ± 0.58$^{a}$ | NI | 16.0±1.0$^{c}$ | NI | 21.0±1.0$^{b}$ |
| *S. epidermidis* ATCC 35984 | 39.7±0.58$^{b}$ | ≥ 44$^{a}$ | NI | 12.7±0.58$^{d}$ | 9.0±1.0$^{e}$ | 24.3±0.58$^{c}$ |
| *S. epidermidis* ATCC 14990 | 29.7±0.58$^{c}$ | ≥ 44$^{a}$ | 20.7±0.58$^{e}$ | 19.7±0.58$^{e}$ | 38.7±0.58$^{b}$ | 23.7±0.58$^{d}$ |

Diameter of inhibition zones includes diameter of discs (5 mm); Values represent mean of triplicate±standard deviation (n=3) in mm; NI: No inhibition; Values ≥ 44 indicates measurement of radius x 2 because of too large and overlapping ZOI; N1: NSS oil sample 1; N2: NSS oil sample 2; All antibiotics were tested at 1 mg/mL; Different letter superscript between columns means significantly different ($p<0.05$).

**Table 2. Antibacterial activity of undiluted samples using well diffusion assay**

| | N1 | N2 | Streptomycin | Ampicillin | Cloxacillin | Chloramphenicol |
|---|---|---|---|---|---|---|
| *E.coli* (clinical isolate) | NI | NI | 12.3±0.58$^{b}$ | NI | NI | 18.3±0.58$^{a}$ |
| *E.coli* ATCC 25922 | NI | NI | 28.3±0.58$^{a}$ | 16.3±0.58$^{b}$ | NI | 29.7±0.58$^{a}$ |
| *Proteus* sp. (clinical isolate) | 9.3±0.58$^{d}$ | 11.0±0.0$^{c}$ | 21.7±0.58$^{a}$ | NI | NI | 18.3±0.58$^{b}$ |
| *P. mirabilis* ATCC 12453 | 23.0±1.0$^{e}$ | 39.7±0.58$^{a}$ | 28.3±0.58$^{d}$ | 29.3±0.58$^{cd}$ | 30.0±0.0$^{bc}$ | 31.3±0.58$^{b}$ |
| *Pseudomonas* sp. (clinical isolate) | NI | NI | NI | NI | NI | NI |
| *P. aeruginosa* ATCC 27853 | 13.3±0.58$^{d}$ | 23.7±0.58$^{b}$ | 26.3±0.58$^{a}$ | 17.7±0.58$^{c}$ | 16.7±0.58$^{c}$ | 24.7±0.58$^{b}$ |
| *Klebsiella* sp. (clinical isolate) | NI | NI | 17.0±0.0$^{a}$ | NI | NI | 9.0±0.0$^{b}$ |
| *Streptococcus* sp. (clinical isolate) | 11.7±0.58$^{c}$ | 23.0±1.0$^{ab}$ | NI | 22.3±0.58$^{b}$ | NI | 24.7±0.58$^{a}$ |
| *S. epidermidis* ATCC 35984 | 38.3 ± 0.58$^{b}$ | ≥ 44a | NI | 13.7±0.58$^{d}$ | 13.3±0.58$^{d}$ | 30.3±0.58$^{c}$ |
| *S. epidermidis* ATCC 14990 | 21.7±0.58$^{e}$ | 33.7±0.58$^{b}$ | 23.3±0.58$^{d}$ | 20.3±0.58$^{e}$ | 41.3±0.58$^{a}$ | 27.0±1.0$^{c}$ |

Diameter of inhibition zones includes diameter of discs (5 mm); Values represent mean of triplicate±standard deviation (n=3) in mm; NI: No inhibition; Values ≥ 44 indicates measurement of radius x 2 because of too large and overlapping ZOI; N1: NSS oil sample 1; N2: NSS oil sample 2; All antibiotics were tested at 1 mg/mL; Different letter superscript between columns means significantly different ($p<0.05$).

**Table 3. Other biological activities of NSS oil**

| Samples | NO scavenging | Elastase inhibition | Tyrosinase inhibition | Melanin inhibition | | Anticancer | |
|---|---|---|---|---|---|---|---|
| | | | | Intracellular | Extracellular | MCF-7 cell line inhibition | HeLa cell line inhibition |
| | IC50 (µg/mL) | | | | | | |
| N1 | 176.8±3.2$^{a}$ | NIA | 544.6±1.915$^{b}$ | NIC | NIC | NID | NID |
| N2 | 148.1±5.8$^{b}$ | NIA | NIB | NIC | 47.83±1.680$^{a}$ | NID | 375.2± 9.9$^{b}$ |
| L-Ascorbic acid | 66.4±1.9$^{c}$ | NT | NT | NT | NT | NT | NT |
| Ursolic acid | NT | 4.27±0.65 | NT | NT | NT | NT | NT |
| Kojic acid | NT | NT | 2.849±4.469$^{a}$ | NT | NT | NT | NT |
| Arbutin | NT | NT | NT | 99.57±1.998 | 99.57±1.998$^{b}$ | | |
| Actinomycin D | NT | NT | NT | NT | NT | 0.0075±3.9 | 0.0022±3.4$^{a}$ |

Values represent mean of triplicate ± standard deviation (n=3) in µg/mL, N1: NSS oil sample 1; N2: NSS oil sample 2; Different letter superscript (lowercase) within column means significantly different ($p<0.05$). IC50: Fifty percent inhibitory concentration,

A: No inhibition at the highest concentration tested of 250 µg/mL

B: No inhibition at the highest concentration tested of 1000 µg/mL

C: No inhibition at the highest concentration tested of 500 µg/mL

D: No inhibition at the highest concentration tested of 400 µg/mL

NI: No inhibition

NT: Not tested

in susceptibility to the tested samples. In general, the ATCC strains were found to be more susceptible compared to the clinical isolates. Overall, N2 was more effective than N1, exerting significantly ($p<0.05$) greater antibacterial activity against *P. mirabilis* ATCC 12453, *P. aeruginosa* ATCC 27853, *Streptococcus* sp. and *S. epidermidis* (ATCC 35984 and ATCC 14990) compared to the reference antibiotics tested, which were found to be ineffective against several tested bacteria (Table 1 and Table 2). For instance, 6 out of 10 bacterial strains were resistant to cloxacillin while 4 strains were resistant to ampicillin. On top of that, the *Pseudomonas* sp. (clinical isolate) was resistant to all 4 antibiotics. The effectiveness of NSS oil against the tested bacteria in the present study is in conformity with the findings of previous studies (Arici et al 2005; Salman et al 2008). However, in contrast to the sensitivity of *Klebsiella* sp. to NSS as observed in our study, the study of Salman et al (2008) showed that NSS oil was not effective against ten multi-drug resistant strains of *K. pneumoniae*. Other *Klebsiella* sp. such as *K. planticola* were also found to be resistant to the oil (Shafi et al 2009). Additionally, resistance to *E. coli* was observed by Salman et al (2008) in contrast to the findings of the current study. This variation in activity can be due to several factors including geographical regions, plant growing conditions, extraction techniques, processing, refining, and purification of the crude oil which may destroy the antibacterial compounds of the oil.

Comparison of the two antibacterial assays revealed significant differences such that the NSS oil samples displayed significantly greater ZOI ($p<0.05$) against all tested bacteria using the disc diffusion method except *Proteus* sp. (clinical isolate) for which the well diffusion assay was found to be better (Fig. 1). For instance, no ZOI was observed in well diffusion assay against the two tested *E.coli* strains, *Pseudomonas* sp. and *Klebsiella* sp. while a small ZOI in the range 7.3-10.3 mm was observed using disc diffusion method. The higher activity of the oil in disc diffusion compared to the well diffusion assay in our study could be due to the direct contact of the disc (impregnated with oil) with the bacteria, indicating a shorter distance for diffusion of the components of the oil through the medium. In contrast, in the well diffusion assay, the bioactive compounds need to diffuse through a longer distance from the bottom of the well to reach the bacteria on the surface of the agar.

**Antioxidant activity**

The antioxidant activity of the tested NSS oil in terms of their scavenging activity against NO radical are shown in Table 3. Among the tested samples, N2 ($IC_{50}$=148.1 µg/ml) displayed significantly greater scavenging activity than N1 ($IC_{50}$=176.8 µg/ml) ($p<0.05$). However, the two NSS oil were found to exhibit significantly lower scavenging activity compared to the positive control, L-Ascorbic acid ($IC_{50}$=66.4 µg/ml) ($p<0.05$). A previous study by Zaher et al (2008) observed a lower $IC_{50}$ value of 110 µg/ml for NSS aqueous extract, hence greater scavenging activity compared to the present study. In addition, NSS aqueous extract was found to exhibit an inhibitory effect on NO production by murine macrophages (Mahmood et al 2003). However, it should be highlighted that few studies have investigated the NO radical scavenging activity of NSS compared to other antioxidant assays such as DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging (Goga et al 2012; Haron et al 2014; Feroz and Uddin 2016), indicating the necessity of conducting more antioxidant assays to obtain the complete antioxidant profile of NSS.

**Elastase inhibitory activity**

The inhibitory effect of NSS oils on elastase activity are displayed in Table 3. At the highest concentration tested (250 µg/ml), no inhibition was observed in contrast to the positive control, ursolic acid, which displayed an $IC_{50}$ value of 4.27 µg/ml. On the contrary, Kacem and Meraihi (2006) found that the essential oil of Algerian NSS showed dose-dependent inhibition on human neutrophil elastase. An increase in activity was observed from 410 µg/ml (about 20% inhibition) to 5800 µg/ml (total inhibition). Therefore, a concentration higher than that used in the present study might show potential antielastase activity which needs to be confirmed by future studies.

**Tyrosinase inhibitory activity**

The tyrosinase inhibitory activity of the two NSS oil samples are presented in Table 3. At the highest concentration tested, 1000 µg/ml, N2 did not show any inhibitory activity. On the other hand, N1 inhibited 50% of the enzyme at a concentration of 544.6 µg/ml, but was less effective compared to the positive control kojic acid ($IC_{50}$=2.849 µg/ml). Few studies have demonstrated the antityrosinase activity of NSS. For instance, an inhibition of 49.6 % was observed by the chloroform: methanol (2:1) extract of NSS at 500 µg/ml (Subramanian and Sahithya 2016). In addition, Muddathir et al (2017) found that the methanolic NSS extract (500 µg/ml) exhibited tyrosinase inhibition of 15.73 % and 17.64 % to L-tyrosine and L-DOPA, respectively, while no inhibition was observed at 125 µg/ml. Interestingly, the study of Sekeroglu et al (2012) revealed a lower active concentration of 50 µg/ml, showing a dose-dependent tyrosinase inhibitory activity.

**Melanin inhibitory activity**

The $IC_{50}$ values obtained in the melanin inhibition assay are shown in Table 3. The NSS oil samples displayed no inhibition on intracellular melanin synthesis at the highest tested concentration (500 µg/ml). In contrast, although N1 was ineffective against extracellular melanogenesis at the highest tested concentration (500 µg/ml), a 50% inhibition was observed by sample N2 at a concentration of 47.83 µg/ml, which was more effective compared to the positive control arbutin ($IC_{50}$=99.57 µg/ml). Compared to previous studies, the antimelanogenic activity of NSS oil observed in the present study was found to be in agreement with that of Lee et al (2011) who found that NSS oil caused a reduction in melanin production up to 86 % at a concentration of 10

mg/ml. Although sample N1 showed no antimelanogenic activity, it should be noted that its tyrosinase activity observed in the present study indicates its indirect role as an antimelanogenic agent due the involvement of the tyrosinase enzyme in melanogenesis (Aumeeruddy et al 2017). It is also important to note that NSS is used as an ingredient in various cosmetic skin products including soap, cream, shampoo, and has been found to possess cosmeceutical properties including anti-hair loss, anti-psoriatic, anti-acne vulgaris, anti-vitiligo, and wound healing properties (Sudhir et al 2016; Eid et al 2017).

**Anticancer activity**

As shown in Table 3, although NSS oil N1 displayed no anticancer activity on HeLa cells at the highest concentration tested (400 µg/ml), N2 was effective with an $IC_{50}$ value of 375.2 µg/ml. Nonetheless, the positive control actinomycin D ($IC_{50}$=0.0022 µg/ml) showed higher activity compared to N2. The cytotoxicity of NSS against HeLa cells have also been observed by various studies. For instance, the methanolic, n-Hexane, and chloroform extracts of NSS were found to display $IC_{50}$ values of 2.28 µg/ml, 2.20 µg/ml and 0.41 ng/ml, respectively, inducing apoptosis in HeLa cells (Shafi et al 2009). The ethanol extract was also found to inhibit proliferation and colony formation, and induce apoptosis in HeLa cells. The apoptotic induction was exerted through the release of mitochondrial cytochrome c, increase of the Bax/Bcl-2 ratio, activation of caspases (3, 8, and 9) and cleavage of poly (ADP-ribose) polymerase. Also, it modulated the expression levels of cell cycle-related proteins such as c-Myc, hTER, cyclin D1, CDK-4, p53, and p21 (Elkady 2012).

On the other hand, the two NSS oils tested in the present study showed no inhibitory effect on MCF-7 cell line at a highest tested concentration of 400 µg/ml, which was in disagreement with several studies. For instance, the study of Baharetha et al (2013) revealed that among 12 supercritical carbon dioxide (SC-$CO_2$) extracts, the extract prepared at higher temperature (60 °C) and lower pressure (2500 psi) showed selective antiproliferative activity against MCF-7 cells with an $IC_{50}$ value of 53.34 µg/ml, and caused significant apoptosis in the cell line by activating caspase 3/7 and 8. Another study (Farah 2005) showed that the ethanolic extract of NSS exerted more potency than the aqueous extract against MCF-7 cells in the presence and absence of $H_2O_2$. In addition, the cytotoxicity of doxorubicin was also enhanced by co-administration with a nanoemulsion of NSS oil (Mahmoud and Torchilin 2013). It is important to highlight that the NO scavenging activity of NSS oil observed in the present study can prevent the reaction of NO with superoxide ($O_2^{\cdot-}$) to form peroxynitrite (ONOO–), a much more powerful oxidant which can be involved in the aetiology of cancer due to its genotoxicity and damages caused to biomolecules (Pacher et al 2007).

**Table 4. Qualitative phytochemical compositions of NSS oils**

| | Samples | |
|---|---|---|
| Phytochemicals | N1 | N2 |
| Alkaloids | + | + |
| Phenols | + | + |
| Flavonoids | + | + |
| Saponins | + | + |
| Steroids | + | + |
| Anthraquinones | + | + |

Note: N1: NSS oil sample 1; N2: NSS oil sample 2

**Table 5. Quantitative phytochemical composition of NSS oils**

| Sample | Total phenolic (µg GAE/g) | Total flavonoid (µg RE/g) | Total tannin (µg CE/g) |
|---|---|---|---|
| N1 | 769.44±5.63[a] | 58.56±1.95[a] | 754.10±15.01[b] |
| N2 | 530.1±12.98[b] | 38.71±1.65[b] | 1369.61±14.82[a] |

Values represent mean of triplicate ± standard deviation (n=3), N1: NSS oil sample 1; N2: NSS oil sample 2; Different letter superscript within columns means significantly different ($p<0.05$). CE, Catechin equivalent; GAE, Gallic acid equivalent; RE, Rutin equivalent

**Phytochemical composition**

Preliminary phytochemical screening revealed the presence of all tested phytochemicals in NSS oil including alkaloids, phenols, flavonoids, saponins, steroids, and anthraquinones (Table 4). Regarding the quantitative phytochemical composition (Table 5), variations were observed among the tested samples such that the TPC of N1 (769.44 µg GAE/g) was found to be significantly greater compared to N2 (530.1 µg GAE/g) ($p<0.05$). A similar pattern was observed for TFC (N1=58.56 µg RE/g; N2=38.71 µg RE/g). In contrast, N1 was found to contain lower TTC (754.10 µg CE/g) compared to

**Table 6. Correlation between TPC, TFC, TTC, colour, and the observed biological activities**

| Assay | TPC | TFC | TTC | L* | a* | b* |
|---|---|---|---|---|---|---|
| NO scavenging activity | -1.000 | -1.000 | 1.000 | -1.000 | 1.000 | 1.000 |
| Tyrosinase inhibitory activity | 1.000 | 1.000 | -1.000 | 1.000 | -1.000 | -1.000 |
| Extracellular antimelanogenic activity | -1.000 | -1.000 | 1.000 | -1.000 | 1.000 | 1.000 |
| HeLa cell inhibitory activity | -1.000 | -1.000 | 1.000 | -1.000 | 1.000 | 1.000 |

TFC, Total flavonoid content; TPC, Total phenolic content. L* represents lightness, a* measures the degree of red (+a*) or green (-a*) colours and b* parameter indicates the degree of the yellow (+b*) or blue (-b*) colours.

N2 (1369.61 µg CE/g). In fact, a strong positive correlation (R=1.000) was observed between TTC and the antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity of NSS oil, while TPC and TFC (R=1.000) were positively correlated with antityrosinase activity (Table 6). Compared with previous studies, the TPC of NSS in the current study was observed to be lower compared to the range 0.96-7.60 mg GAE/g as found by Haron et al (2014) and Lutterodt et al (2010).

These variations could be attributed to the extraction techniques as well as other reasons mentioned previously, resulting in variations in the chemical compositions of the oil. Although the antibacterial, antioxidant, extracellular antimelanogenic, and HeLa cell line inhibitory activity of sample N2 was found to be higher than N1, N2 displayed lower total phenolic and flavonoid content. This might suggest that compounds found in higher concentrations are not necessarily responsible for the total activity. It might be that the different compounds in small concentrations act synergistically or specific phenolic and flavonoid compounds with stronger bioactivities are present in higher concentration.

**Table 7. Physicochemical properties of NSS oil**

| | | N1 | N2 |
|---|---|---|---|
| pH | | ND | ND |
| Color | L* | 66.48±0.64$^a$ | 60.48±2.18$^b$ |
| | a* | 9.87±0.49$^b$ | 13.07±1.30$^a$ |
| | b* | 46.38±0.19$^b$ | 50.42±2.47$^a$ |
| Total soluble solids (˚Brix) | | 73.5±1.14$^a$ | 73.3±0.49$^a$ |
| Density (g/ml) | | 0.85±0.01$^b$ | 0.89±0.00$^a$ |

Values represent mean of triplicate ± standard deviation (n=3), ND: Not detected; N1: NSS oil sample 1; N2: NSS oil sample 2; (-): not detected; Different letter superscript between columns means significantly different ($p<0.05$). L* represents lightness, a* measures the degree of red (+a*) or green (-a*) colours and b* parameter indicates the degree of the yellow (+b*) or blue (-b*) colours.

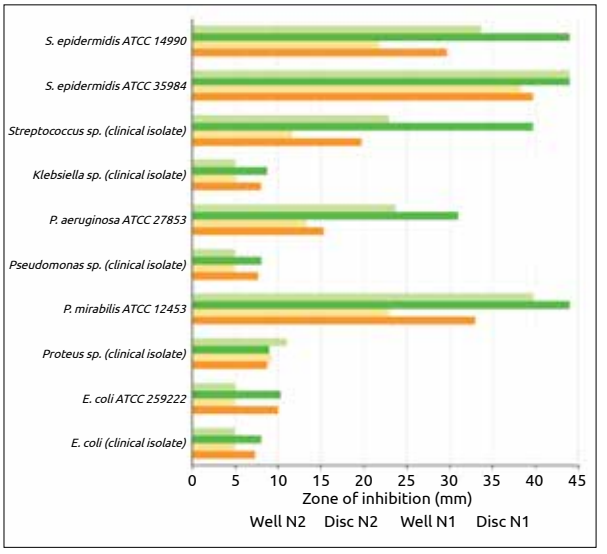


**Figure 1**. Mean ZOI for disc diffusion and well diffusion assay

Diameter of ZOI includes diameter of disc and well (5 mm); ZOI of 5 mm indicates no inhibition; N1: NSS oil sample 1; N2: NSS oil sample 2

For instance, the higher concentration of tannins observed in N2 compared to N1, might explain the higher antibacterial activity of N2. In fact, tannins isolated from several plants have been found to possess significant antimicrobial activity (Ho et al 2001; Jada et al 2014). Moreover, thymoquinone (TQ) has been found to be a major contributor to the biological activities of NSS including antimicrobial activity (Shohayeb and Halawani 2012). In addition, TQ (about 60% inhibition) and thymohydroquinone (35% inhibition) at 2 µg/ml, were more effective than NSS oil in suppressing melanin production (Lee et al 2011). The mechanisms by which TQ exhibits its anticancer activity against MCF-7 and HeLa cells have also been reported by several studies (Alobaedi et al 2017; Cakir et al 2016; Rajput et al 2013; Woo et al 2011; Yazan et al 2009).

However, it is important to highlight that although TQ has been found to the major contributor to the antioxidant activity of NSS, other constituents, mainly those of the essential oil fraction, including carvacrol, thymol, t-anethole, γ-terpinene, 4-terpineol, p-cymene, β-pinene, and α-thujene, have also been found to be responsible ( Ahmad and Beg 2014; Burits and Bucar 2000; Kazemi 2014). Additionally, carvacrol was found to be the most bioactive compound in inhibiting human neutrophil elastase with a very low $IC_{50}$ value (12 µM), followed by carvone ($IC_{50}$=14 µM), p-Cymene ($IC_{50}$=25 µM), and TQ ($IC_{50}$=30 µM) (Kacem and Meraihi 2006). In addition, thymohydroquinone was found to display higher tyrosinase inhibitory activity (68.82% inhibition at 120 µg/ml) compared to TQ (8.24% inhibition at 166.67 µg/ml) (Lee et al 2011). Therefore, the total bioactivity of NSS is not likely to be due to the presence of one main compound but rather due to its plethora of phytochemicals. These bioactive compounds may act in combination to produce synergistic or additive effect which need to be confirmed by future studies.

**Physicochemical properties**

The physicochemical properties of the tested samples including TSS, colour, and density are shown in Table 7. No significant difference was observed in the TSS (˚Brix: N1= 73.5; N2=73.3) of the two NSS oil tested ($p>0.05$). On the other hand, significant difference was observed in the density of the tested samples such that N2 (0.89 g/mL) exhibited a higher density than N1 (0.85 g/mL) ($p<0.05$). The density of NSS oil was found to be close to that reported by the study of Zzaman et al (2014) (0.93-0.98 g/mL).

Comparison of the colour of the tested samples revealed that N2 displayed significantly lower L* value, higher a* and b* values (L*=60.48, a*=13.07, b*=50.42) than N1 (L*=66.48, a*=9.87, b*=46.38) ($p<0.05$). This may suggest the presence of more yellow pigments (e.g. carotenoids) in N2, which may

have contributed to its higher antioxidant activity compared to N1. Compared to other studies, the two NSS oil in the current study displayed higher L* and a* value, and lower b* value than Tunisian and Iranian NSS oil (Cheikh-Rouhou et al 2007). This discrepancy might be due to difference in seed variety or extraction technique employed. In fact, the study of Zzaman et al (2014) showed that screw pressed NSS oil at different temperatures displayed variations in colour. Interestingly, a* and b* values were positively correlated (R=1.000) with the antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity, while L* was positively correlated with the antityrosinase activity (R= 1.000) (Table 6).

## CONCLUSION

From the present investigation, it was found that NSS oil possess major bioactive phytochemicals and significant biological activities including antibacterial, antioxidant, antityrosinase, antimelanogenic, and anticancer activity (against HeLa cells). We observed variations between the two NSS oil samples such that N2 displayed higher antibacterial, antioxidant, extracellular antimelanogenic, and HeLa cell inhibitory activity compared to N1, which showed higher antityrosinase activity. The higher antibacterial activity of N2 when compared to standard antibiotics in the present study highlights its potential role in effectively slowing the evolution of antibiotic resistance in bacteria. We also found that TTC was positively correlated with most observed activities. The two NSS oil samples also displayed significant variation in physicochemical properties including density and colour whereby the a* and b* values were positively correlated with most observed bioactivities. Nonetheless, it has been argued that *in vitro* models inherently bear limitations and fail to replicate the precise cellular and metabolic conditions of an organism. Therefore, it is necessary to validate the observed biological activities together with toxicological analysis *in vivo* and clinically to obtain the therapeutic dose for the treatment and/or management of communicable and noncommunicable diseases.

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