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Chemical composition and repellency of *Nigella sativa* L. seed essential oil against *Anopheles gambiae* sensu stricto

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ABSTRACT

Malaria which is caused by the *Plasmodium* parasite and transmitted through bites of infected female *Anopheles mosquitoes* is an important public health concern in Africa. Insect repellents are commercially available but most of the synthetic repellents have adverse effects to the user as well as the environment. Previous studies have shown that *Nigella sativa* L. seed extracts have insecticidal and insect repelling activity. The objective of the present study was to evaluate the repellence efficacy of essential oil of *Nigella sativa* L. seeds on *Anopheles gambiae* sensu stricto and to determine the chemical composition of the essential oil. The repellence activity of the essential oil was dose-dependent and comparable to that of DEET (*N*,*N*-diethyl-m-toluamide). The repellence of the oil was 98.81 and 100% at concentrations of 0.01 and 0.1 g/mL, respectively. GC-MS analysis showed the major components of the essential oil to be *p*-cymene (34.67%), α-thujene (11.55%), *trans*-4-methoxythujane (5.81%), β-pinene (4.66%), methylcyclohexane (3.11%), α-pinene (2.82) and longifolene (2.55%). The repellence activity of *N. sativa* seed oil against *An. gambiae* can be attributed to the presence of α-pinene, *p*-cymene and longifolene. These findings have confirmed that the essential oil of *N. sativa* seeds contains compounds that repel *An. gambiae* and therefore can be used to control spread of malaria through prevention of mosquito bites.

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1. Introduction

Malaria is an important public health concern caused by the *Plasmodium* parasite, which is transmitted to people through the bites of infected female *Anopheles* mosquitoes (WHO, 2015). Malaria occurs mostly in poor tropical and subtropical areas of the world. In many of the countries affected by malaria, it is a leading cause of illness and death. In 2016, malaria caused an estimated 216 million clinical episodes and 445,000 deaths worldwide with about 90% of deaths occurring in African countries (WHO, 2017). One of the best ways to deal with this global enemy is to prevent mosquito bites by using physical and chemical barriers, treatment of fabric with toxicants, and the use of topical repellents (Barnard and Xue, 2004).

Several synthetic mosquito repellents are commercially available to consumers worldwide. Most of the repellents contain DEET (*N*,*N*-diethyl-m-toluamide), which has broad-spectrum activity and effectively repels most mosquitoes, biting flies, chiggers, fleas, and ticks (Fradin and Day, 2002). DEET is formulated in aerosols, pump sprays, lotions, creams, liquids, sticks, roll-ons, and impregnated towelettes, with concentrations ranging from 5 to 100% (Keziah et al., 2015). Mosquitoes have developed resistance to most synthetic repellents which in turn possess public health concerns as they trigger allergy, skin and eye irritations and respiratory disorders (Ogbonnia et al., 1990). These repellents also contaminate food, water and pollute air as well as release chlorofluorocarbons

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(CFCs) that deplete ozone layer (Lutz et al., 2014). The use of medicinal plants in disease and pest control has been practiced for several thousands of years. Herbal medicine plays an important role in meeting the primary healthcare needs of the population in most developing countries (Ochieng et al., 2013; Ochung et al., 2015; Jeruto et al., 2017; Ganesan and Xu, 2017; Nunes and Miguel, 2017; Mohammadhosseini et al., 2019; Wansi et al., 2019). Previous studies have shown that bioactive compounds in plants have the capacity to combat numerous types of diseases (Opiyo et al., 2011a; 2011b; Ochieng et al., 2017; Ochung et al., 2018; Wansi et al., 2018; Njoroge and Opiyo 2019a; 2019b). Extracts from plants species including *Annona mucosa* Jacq, *Ocimum Kilimandscharicum* Guerke, *Trachyspermum ammi* (Linn) Sprague, *Warburgia ugandensis* Sprague have been shown to exhibit repellent and toxic effects against insects (Pandey et al, 2009; Manguro et al., 2010; Opiyo et al., 2015; Opiyo, 2019; Makenzi et al., 2019a; 2019b). Several plants including *Ocimum gratissimum* L., *Lantana camara* L., *Cassia obstusifolia* L., *Hyptis suaveolens* L., *Striga hermonthica*, *Sumerian* and *Eucalyptus citriodora* Hook have been studied as possible mosquito repellents and the findings have revealed the existence of natural repellents (Esimone et al., 2011; Maia et al., 2011; Keziah et al., 2015). Insect repellents of plant origin are preferred since they are more friendly to both the user and the environment compared to synthetic medicines (Maia et al., 2011; Adib-Hajbaghery and Rafiee, 2018). *Nigella sativa* L. (also called black cumin, or black seed) is an annual herb of the Ranunculaceae family and is cultivated in various parts of the globe (Farooqi et al., 2005). *N. sativa* L. seeds are used in Iranian folk and traditional medicines to treat asthma, fever, cough, eczema, headache, rheumatism, influenza and bronchitis (Hajhashemi et al., 2004). Previous studies have shown that extracts and oils from the plant seeds have anti-inflammatory, antimicrobial and antioxidant activities (Haseena et al., 2015; Dinagaran et al., 2016; Mohammed et al., 2019). Extracts from the plant also exhibited insecticidal and insect repelling activity against *Amblyomma americanum* Linnaeus, *Tribolium castaneum* Herbst, *Tuta absoluta* Meyrick, *Aedes aegypti* Linnaeus, *An. stephensi* Meigen and *Culex quinquefasciatus* Say (Adil et al., 2015; Raj et al 2015; Wijayanti et al., 2019). Several bioactive compounds have been reported from the *N. sativa* L. seed including thymoquinone, nigellone, thymohydroquinone, dithymoquinone, *p*-cymene and α-pinene (Haseena et al., 2015). Thymoquinone (TQ) is a chief bioactive constituent of *N. sativa* seed oil and holds promising pharmacological properties against several diseases. It exhibited antioxidant, anti-inflammatory, anticancer, and other important biological activities (Costa et al., 2015). Bioactivity and chemical composition of plant vary with species, geographical location, plant part, maturity and extraction method (Kokoska et al., 2008; Botnick et al., 2012). The oils obtained by hydrodistillation (HD) and steam distillation (SD) were dominated by *p*-cymene, whereas the major constituent identified in both steam distillation of crude oil obtained by solvent extraction (SE-SD) and steam distillation of crude oil obtained by supercritical fluid extraction (SFE-SD) was thymoquinone (Kokoska et al., 2008). Analysis of *N. sativa* essential oil from Morocco revealed the main components as *p*-cymene (33.8%) and thymol (26.8%) with only small amounts of thymoquinone (3.8%) (Moretti et al., 2004), whereas analysis of Indian sample gave rise to the identification of 9-eicosyne 63.0%, linoleic acid (13.5%), palmitic acid (9.7%) (Dinagaran et al., 2016), thymol (19.1%), α-phellandrene (14.9%), camphor (12.1%), borneol (11.3%) and carvacrol (8.6%) (Raj et al., 2015). The objective of the present study was to evaluate the repellence efficacy of essential oil from *N. sativa* seeds against *Anopheles gambiae* sensu stricto and to determine the chemical composition of the oil.

2. Experimental

2.1. Plant material

Dry *N. sativa* seeds imported from India were purchased from Parklands Market in Kenya. The sample was cleaned and freed from dust and foreign material and then ground into coarse powder using an electric house-hold spice grinder.

2.2. Extraction of essential oil

The ground *N. sativa* seeds (125 g) were transferred into a clean flask and 500 mL of distilled water was added. Ten grams of sodium chloride was added to the mixture to reduce the foaming during boiling. Oil extraction was done by hydrodistillation method using the Clevenger-type apparatus (Abdellatif and Hassani, 2015). The apparatus was set-up and the mixture was heated at 100 °C for 3 hours. The oil was separated from the aqueous phase using a separating funnel to give 0.68 g of oil which was stored in amber-colored vial at -4 °C for phytochemical analysis and bio-assays.

2.3. Experimental insects

Anopheles gambiae sensu stricto were obtained from colonies reared in the insectaries at International Centre of Insect Physiology and Ecology (ICIPE) Nairobi, Kenya that are reared according to the WHO protocol (WHO, 1996). *Anopheles gambiae* eggs were hatched by simultaneously flooding the moist filter paper platforms. Rearing was carried out in the insectary maintained at 27-28 °C and approximately 80% humidity on a 12h/12h light and darkness cycle and maintained at optimal larval concentrations to avoid possible effects of competition. The larvae were fed on ground baby fish food while adults were offered a fresh 10% (w/v) sucrose solution meal daily and on hamsters as a source of blood meals when required to produce eggs.

2.4. *Anopheles gambiae* repellency assay

Tests were carried out according to WHO protocol (1996) and Barnard (2005). Repellency assays were

done against 5-7 days old females of *An. gambiae* that had been starved for 18 hours but previously fed on 6% glucose solution (Omolo et al., 2004). The essential oil was tested at concentrations of 10, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} g/mL. The tests were performed on 6 human adults (18 yrs ≤ age ≤ 50 yrs). Each test used different set of *An. gambiae*. The participants had no contact with lotions, perfumes oil or perfumed soaps on the day of the experiment. Insect bite cream was provided to the participants in case of any minor bites and associated irritations. Test solutions (1.0 mL) were dispensed on one of the forearms of a volunteer from wrist to the elbow covering an area of 500 cm^2. The rest of the hand was covered with a glove. Acetone (25% in water) was dispensed on the other forearm to serve as control. The control arm was first introduced into the cage immediately after releasing the 50 experimental insects and kept there for 3 minutes. The number of insects that landed on control arm during the test was recorded. The treated arm was then introduced into the cage for the same period of time and the number of landing insects recorded. The different concentrations (0.00001, 0.0001, 0.001, 0.01 and 0.1 g/mL) of the sample and DEET were tested starting with lowest concentrations. Percentage protective efficacy (PE) was calculated using the formula (Eqn. 1):

$$PE = (C-T/C) \times 100\% \quad \text{(Eqn. 1)}$$

Where C and T are the mean numbers of insects that landed on the control and the test arm, respectively (Yap et al., 1998).

2.5. Determination of chemical composition of essential oil

Chemical composition of the essential oil of *N. sativa* seeds was determined using GC and GC-MS techniques (Thollet et al., 2006). Initial analysis was carried out on a Hewlett Packard 5890 series II GC system, equipped with FID coupled to HP 3393A series II integrator. A HP-5 capillary column of 30 m x 0.25 mm and 0.25 µm thickness was used. The carrier gas was nitrogen at a flow rate of 0.7 mL min^{-1}. The temperature was maintained at 50 °C for 5 minutes, then increased at 5 °C per minute up to 280 °C and held for 10 minutes. Identification of essential oil components was carried out on GC-MS HP 8060 series II GC coupled with a VG Platform II mass spectrometer with the following parameters: ionization mode: EI = 70 eV; emission current: 200 µA; temperature of the source: 180 °C and multiplier voltage: 300 V. The MS had a scan cycle of 1.5 s (scan cycle of 1 s and inter-delay of 0.5 s) and scan ranges m/z 38-650. Helium was used as the carrier gas and the column temperature, 50 °C (5 min) to 90 °C at 5 °C min^{-1} to 200 °C at 2 °C min^{-1} to 280 °C at 20 °C min^{-1} (held for 20 min). The constituents of essential oil were identified by analysis of their mass spectra and direct comparison with the Institute of Standards Technology libraries 98.1 (NIST) and Wiley Registry of Mass Spectral Data, 8th edition database of library of mass spectra, on the GC-MS equipment. Standard solutions of linear alkanes (C_5-C_{15}) were used for Kovats RI calibration in the GC-MS system.

2.6. Ethical issues

This study was given an ethical approval by Kenyatta University Ethics Review Committee, Kenya with the reference number of KU/R/COMM/51/16. This acceptance was after submitting of the detailed proposal of the research to the Ethics Review Committee for proper study. All volunteers were given written consent forms which they signed in front of a witness who was not a study participant.

3. Results and Discussion

3.1. Repellency of *N. sativa* seed essential oil

Hydro-distillation of the seeds gave 0.54% essential oils, which is similar to yields previously reported (Michelitsch et al., 2004). Repellency of the *N. sativa* seed essential oil at doses of 0.00001, 0.0001, 0.001, 0.01 and 0.1 g/mL were determined against *An. gambiae* and the mean percent repellence are given in Table 1. The repellency of the oil was concentration-dependent and at concentrations of 0.00001 and 0.0001 g/mL, the oil showed 36.97 ± 1.81 and 50.41 ± 2.87% repellence, respectively. At a concentration of 0.1 g/mL, the essential oil was as repellent as DEET, a commercial insect repellent and repelled 100.00% of the test insects. These results are in agreement with previous reports which showed that of *N. sativa* seed essential oil have insecticidal and insect repellent activities (Ahmed, 2007; Raj et al 2015; Wijayanti et al., 2019).

3.2. Chemical composition essential oil of *N. sativa* seeds

GC-MS analysis of *N. sativa* seeds essential oil gave 26 compounds (Fig. 1 and Fig. 2) which were identified by comparing their fragmentation patterns using Institute of Standards Technology libraries 98.1 (NIST) and Wiley Registry of Mass Spectral Data, 8th edition. The major compounds and their relative abundances are given in Table 2. Different classes of compounds were identified having different proportions including monoterpenoid hydrocarbons (59.94%), sesquiterpenoid hydrocarbons (3.30%) and non-terpenoid hydrocarbons (4.90%). The major components were *p*-cymene (34.67%), α-thujene (11.55%), *trans*-4-methoxythujane (5.81%), β-pinene (4.66%), methylcyclohexane (3.11%), α-pinene (2.82%) and longifolene (2.55%). A compound whose retention time is 13.05 minutes with relative abundance of 5.81 could not be identified using the NIST data base and Wiley Registry of Mass Spectral Data used (NIST 2017). Its mass spectrum compared closely with that reported by Wajs et al. (2008) who identified it as *trans*-4-methoxythujane by comparison of the relevant ^{1}H-NMR, and MS spectra of the isolated compound with those of a synthetic product.

The compound's identity was further confirmed by determining its Kovats retention index which was found to be 1105 and was in agreement with previous report (NIST 2017). These findings on chemical composition were in line with previous reports which showed monoterpenes to be the main constituents of *N. sativa* seeds essential oil and that *p*-cymene was the main component of the plant (Moretti et al., 2004, Dinagaran et al., 2016; Mouwakeh et al., 2018). Whereas thymoquinone (TQ) has been reported as one of the main components of *Nigella sativa* seed oil (Costa et al., 2015; Haseena et al., 2015), it was not detected in this study. The differences in chemical composition observed in this study could be explained by the fact that chemical composition of plant species varies greatly depending on geographical location, maturity and extraction method (Costa et al., 2015; Haseena et al., 2015). Apha-pinene repelled house fly, *Musca domestica* (Haselton et al., 2015), while in other studies the compound was larvicidal against *Anapheles subpictus*, *Aedes albopictus* and *Culex tritaeniorhynchus* (Govindarajan et al., 2016). *p*-Cymene showed repellent activity against *Amblyomma americanum* L. and *Aedes aegypti* L. (Carroll et al., 2017) while longifolene was termiticidal and antifeedant against *Reticulitermes speratus* Kolbe (Mukai et al 2017). Therefore, the repellence activity of *N. sativa* seed oil against *An. gambiae* can be attributed to the presence of α-pinene,

Table 1
Percentage repellency of *N. sativa* seed essential oil against *Anopheles gambiae* sensu stricto.

	Concentration in g/mL				
Test material	**0.000**	**0.000**	**0.001**	**0.01**	**0.1**
Essential oil	36.97 ± 1.81	50.41 ± 2.87	84.17 ± 0.78	98.81 ± 1.19	100.00 ± 0.00
DEET	51.11 ± 13.32	86.22 ± 4.51	94.29 ± 3.69	100.00 ± 0.00	100.00 ± 0.00

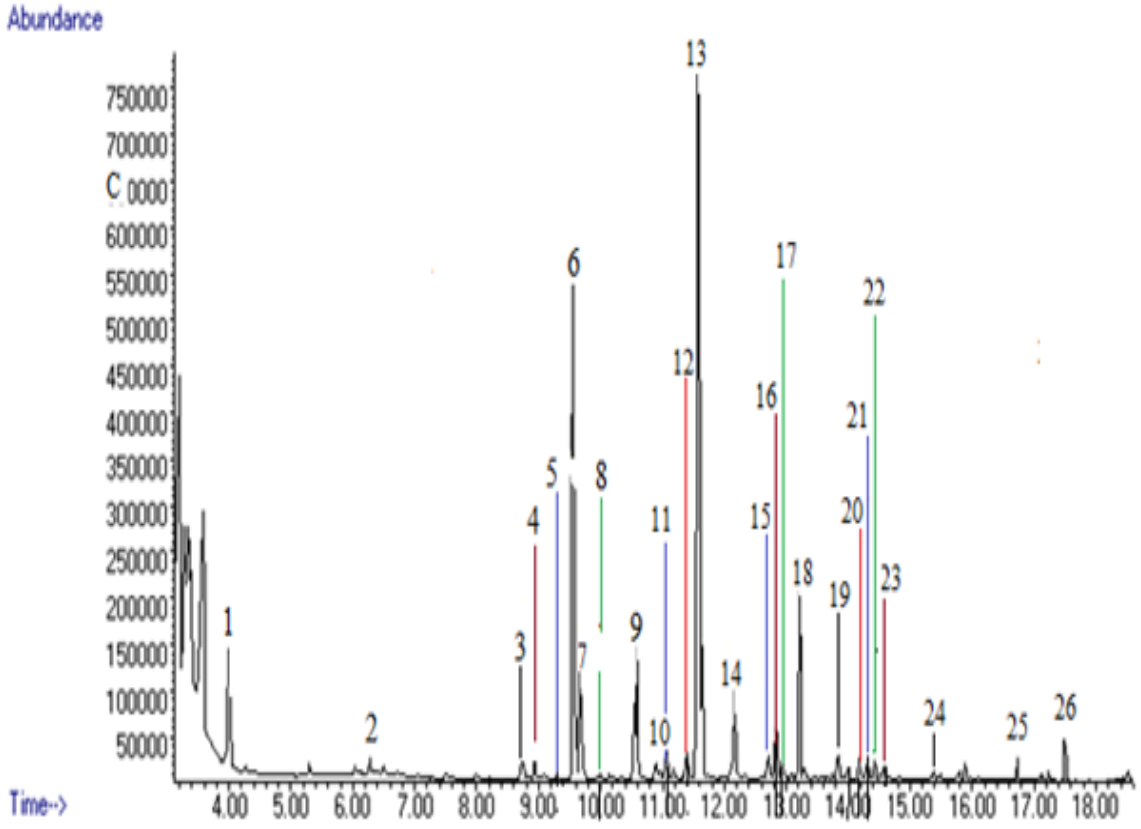

Fig. 1. Gas Chromatogram of seeds essential oil from *N. sativa*.

Table 2
GC-MS chemical constituents of *Nigella sativa* seeds essential oil.

S / no	Chemical name	Retention time (min.)	KI[a]	KI[b]	Relative abundance	Classification
1.	Methylcyclohexane	4.03	723	726	3.11	Non-terpenoid hydrocarbon
2.	Octane	6.07	801	800	0.02	Non-terpenoid hydrocarbon
3.	Styrene	8.57	893	891	0.05	Non-terpenoid hydrocarbon
4.	Nonane	8.75	902	900	0.06	Non-terpenoid hydrocarbon
5.	1,2,3,4,5-Penta-methylcyclopentane	9.13	917	915	0.08	Non-terpenoid hydrocarbon
6.	α-Thujene	9.38	930	930	2.82	Monoterpenoid hydrocarbon
7.	α-Pinene	9.49	935	934	0.06	Non-terpenoid hydrocarbon
8.	Propylbenzene	9.99	958	944	4.66	Monoterpenoid hydrocarbon
9.	β-Pinene	10.41	978	980	0.07	Non terpenoid hydrocarbon
10.	1-Decene	10.75	994	990	0.07	Non terpenoid hydrocarbon
11.	Decane	10.88	1001	1000	0.09	Monoterpenoid hydrocarbon
12.	δ-Carene	11.22	1016	1017	34.67	Monoterpenoid hydrocarbon
13.	*p*-Cymene	11.42	1026	1026	0.26	Non terpenoid hydrocarbon
14.	γ-Terpinene	12	1053	1055	0.15	Non terpenoid hydrocarbon
15.	(*Z*)-3-Undecene	12.16	1061	1068	0.09	Non terpenoid hydrocarbon
16.	(*E*)-4-Undecene	12.79	1091	1091	0.06	Non terpenoid hydrocarbon
17.	(*E*)-5-Undecene	12.94	1098	1099	5.81	Monoterpenoid ether
18.	*trans*-4-Methoxythujane	13.05	1105	1110	0.14	Non terpenoid hydrocarbon
19.	Pentylbenzene	13.68	1158	1158	0.13	Monoterpenoid alcohol
20.	Terpinen-4-ol	14.11	1185	1182	0.11	Non terpenoid hydrocarbon
21.	Dodecene	14.15	1189	1189	0.14	Non terpenoid hydrocarbon
22.	Dodecane	14.29	1201	1200	0.21	Monoterpenoid ketone
23.	β-Cyclocitral	14.42	1210	1216	0.43	Non terpenoid hydrocarbon
24.	Hexylbenzene	15.25	1260	1257	0.75	Sesquiterpenoid hydrocarbon
25.	α-Longipinene	16.57	1366	1351	2.55	Sesquiterpenoid hydrocarbon
26.	Longifolene	17.33	1417	1416	2.55	Sesquiterpenoid hydrocarbon
Monoterpenoid hydrocarbon					**59.94**	
Sesquiterpenoid hydrocarbon					**3.3**	
Non terpenoid hydrocarbon					**4.9**	

[a] The Kovats Retention Indices were calculated relative to a series of *n*-alkanes.
[b] Kovats Retention Indices from literature (Adams, 1995).

p-cymene and longifolene.

4. Concluding remarks

From the results in this study, it is evident that essential oil of *N. sativa* seeds contains compounds that repel *An. gambiae* and therefore can be used to control spread of malaria through prevention of the insect bites. The results further confirm that plant species affects chemical composition which leads to variation in their bioactivities against pathogens and pests. Further studies should be done to determine the mode of action and protection time of the oil. A more reliable method such as use Kovats indices should be used to confirm the identity of the strange compound whose retention time was 13.05 min.

Conflict of interest

The authors declare that there is no conflict of interest.

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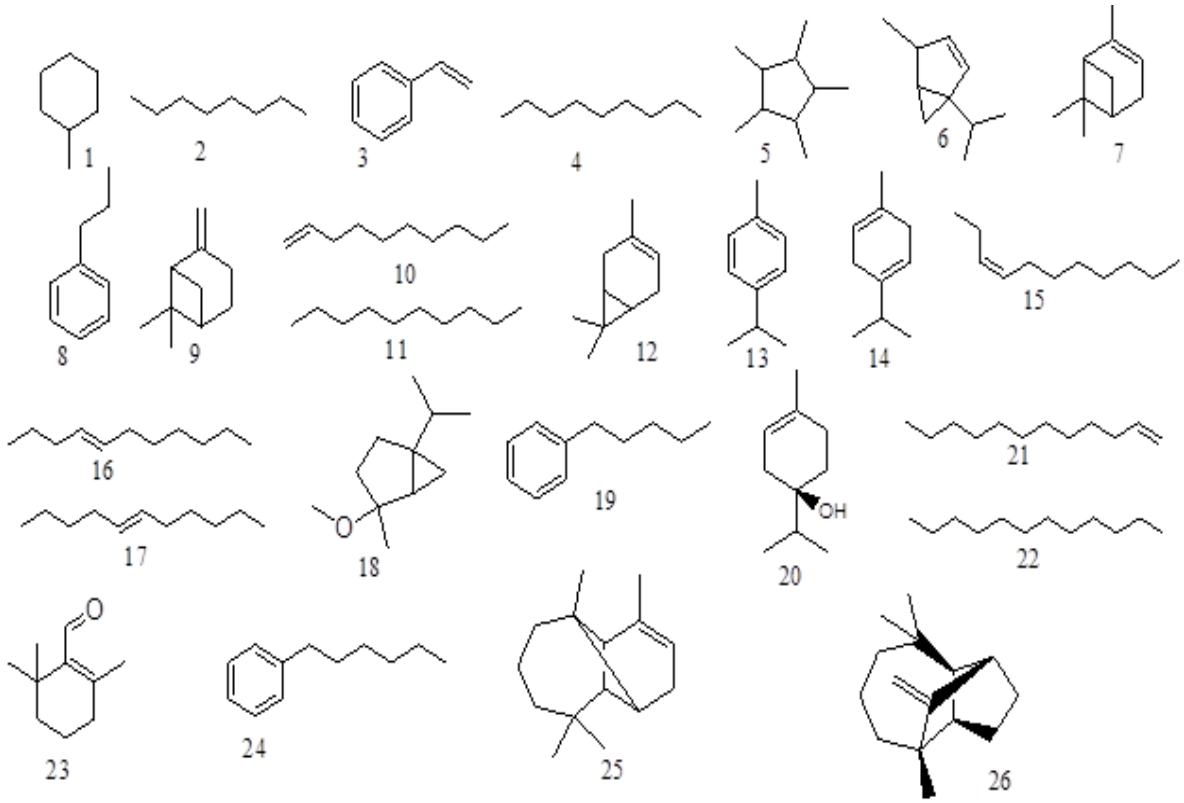

Fig. 2. Molecular structures of the characterize chemical compounds in *Nigella sativa* seed essential oil.

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