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# Journal of Pharmacognosy and Phytotherapy

### Full Length Research Paper

# Analysis of bioactive chemical compounds of *Nigella* sativa using gas chromatography-mass spectrometry

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Phytochemicals are chemical compounds often referred to as secondary metabolites. Twenty eight bioactive phytochemical compounds were identified in the methanolic extract of *Nigella sativa*. The identification of phytochemical compounds is based on the peak area, retention time molecular weight, and molecular formula. Gas chromatography-mass spectrometry (GC-MS) analysis of *Nigella sativa* revealed the existence of the ß-Pinene, D-Glucose,  $6\text{-O-}\alpha\text{-Dgalactopyranosyl}$ , O-Cymene, DL-Arabinose, Trans-4-methoxy thujane, 2-Propyl-tetrahydropyran-3-ol, Terpinen-4-ol,  $\alpha$ - D-Glucopyranoside, O- $\alpha$ -D-glucopyranosyl-(1.fwdarw.3)-ß-D-fruc, Thymoquinone, 2-Isopropylidene-5-methylhex-4-enal, Limonen-6-ol, pivalate, Longifolene, 2-(4-Nitrobutyryl)cyclooctanone, ß-Bisabolene, 1,1-Diphenyl-4-phenylthiobut-3-en-1-ol, Phenol, 4-methoxy-2,3,6-trimethyl, Pyrrolidin-2-one-3ß-(propanoic acid, methyl ester),5-methylene-4 $\alpha$ , Cholestan-3-ol, 2-methylene-,(3ß,5 $\alpha$ ), I-(+)-Ascorbic acid 2,6-dihexadecanoate, 9,12-Octadecadienoic acid (Z,Z)-, methyl ester, 1-Heptatriacotanol, 10,13-Eicosadienoic acid, methyl ester, E,E,Z-1,3,12-Nonadecatriene-5,14-diol, 9-Octadecenamide,(Z), 2H-Benzo[f]oxireno[2,3-E]benzofuran-8(9H)-one,9-[2-(dimethylar, Phthalic acid, decyl oct-3-yl ester, 1,2-Benzenedicarboxylic acid, bis(8-methylnonyl) ester and Stiqmasterol.

**Key words:** Gas chromatography-mass spectrometry, Fourier-transform infrared spectroscopy, *Nigella sativa*, phytochemicals.

#### INTRODUCTION

Nigella sativa L. (Ranunculaceae) is an annual herbaceous plant native to (and cultivated in) Southwest Asia, and cultivated and naturalized in Europe and North Africa (Al-Johar et al., 2008). The seeds and seed oil have been used as a diuretic, appetitizer, hemorrhagic and anti-dandruff therapy in folk medicine (Al-Othman et al., 2006). N. sativa L. commonly known as Kalonji in

Hindi, a member of Ranunculaceae family, also known as the black cumin seeds is one of the most revered medicinal seeds in history. It is an annual aromatic plant and its cultivation is traced back more than 3000 to the kingdom of the Assyrians and ancient Egyptians (Ashraf et al., 2006; Hameed et al., 2015a). *N. sativa* taxonomic classification (Ashraf, 2011), depicts it is a flowering

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dicotyledon plant belonging to family Ranunculaceae under kingdom plantae. Morphology *N. sativa* is an annual medicinal herb, about 30 to 60 cm high (Boskabady et al., 2007; Jasim et al., 2015), with finely divided, linear leaves. The flowers are usually pale blue and white, with 5 to 10 petals. The fruit is a large inflated capsule that composed of 3 to 7 united follicles, each containing numerous black trigonal seeds (Chaudhry and Tariq, 2008; Hameed et al., 2015b).

The black kalonji seeds possess the anthelmintic, insecticidal, antimalarial, antibacterial, antifungal, and antitumor effects. There are also reports that black seeds possess diuretic, carminative, digestive and antiseptic properties (Burits and Burcar, 2000; Ali and Blunden, 2003; Saleh, 2006; Abdulelah and Abidin, 2007; Ali et al., 2008). The seeds have also been used traditionally for centuries in the Middle East, Far East, and some Mediterranean and European countries for the treatment of different ailments, such as diabetes, hypertension, cardiac diseases, hemorrhoids, and sexual diseases and as an abortifacient (Kanter, 2008; Iqbal et al., 2010).

Seeds of *N. sativa* are reported to contain amino acids, carbohydrates, fixed and volatile oils. The yield of black seed fixed oil ranges from 22.0 to 40.35% (Ali and Blunden, 2003; Cheikh-Rouhou et al., 2007; Altameme et al., 2015a). The extracts of *N. sativa* (Black seeds) have been used by patients to suppress coughs, disintegrate renal calculi (Hashem and El-Kiey, 1982), retard the carcinogenic process, treat abdominal pain, diarrhea, flatulence, and polio (Enomoto et al., 2001), exert choleretic and uricosuric activities, anti-inflammatory and antioxidant effects (Mansour et al., 2002; Altameme et al., 2015b). Besides, the essential oil was shown to have antihelminthic, antischistosomal (Mahmoud et al., 2002), antimicrobial (Aboul-Ela et al., 1996), and antiviral.

#### **MATERIALS AND METHODS**

#### Preparation of extract

N. sativa were purchased from local market in Hilla city, middle of Iraq. After thorough cleaning and removal of foreign materials, the seeds were stored in airtight container to avoid the effect of humidity and then stored at room temperature until further use (Hameed et al., 2015c; Hamza et al., 2015). N. sativa seeds are washed with water, dried and ground into powder. N. sativa seeds powder was macerated using methanol for 1 x 24 h. Whatman No.1 filter paper was used to separate the extract of plant. The filtrates were used for further phytochemical analysis. It was again filtered through sodium sulphate in order to remove the traces of moisture (Hussein et al., 2015).

#### Gas chromatography-mass spectrum (GC-MS) analysis

The GC-MS analysis of the plant extract was made in a Agilent 7890 A instrument under computer control at 70 eV. About 1  $\mu$ l of the methanol extract was injected into the GC-MS using a micro syringe and the scanning was done for 45 min. As the compounds

were separated, they eluted from the column and entered a detector which was capable of creating an electronic signal whenever a compound was detected (Imad et al., 2014a; Hameed et al., 2015d). The greater the concentration in the sample, the bigger the signal obtained which was then processed by a computer. The time from when the injection was made (Initial time) to when elution occurred is referred to as the retention time (RT). While the instrument was run, the computer generated a graph from the signal called chromatogram. Each of the peaks in the chromatogram represented the signal created when a compound eluted from the gas chromatography column into the detector (Mohammed and Imad, 2013). The x-axis showed the RT and the yaxis measured the intensity of the signal to quantify the component in the sample injected. As individual compounds eluted from the gas chromatographic column, they entered the electron ionization (mass spectroscopy) detector, where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with a certain mass .The mass/charge (M/Z) ratio obtained was calibrated from the graph obtained, which was called as the mass spectrum graph which is the fingerprint of a molecule (Imad et al., 2014b). Before analyzing the extract using GC-MS, the temperature of the oven, the flow rate of the gas used and the electron gun were programmed initially. The temperature of the oven was maintained at 100°C. Helium gas was used as a carrier as well as an eluent. The flow rate of helium was set at 1 ml/min. The electron gun of mass detector liberated electrons having energy of about 70eV.The column employed here for the separation of components was Elite 1(100% dimethyl poly siloxane). The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. Compounds were identified by comparing their spectra to those of the Wiley and NIST/EPA/NIH mass spectral libraries (Kareem et al., 2015; Imad et al., 2014c).

#### **RESULTS AND DISCUSSION**

Preparation of the extract was done by maceration method. Maceration was done using the appropriate solvent with several times shaking or stirring at room temperature (Rader et al., 2007). Maceration is a method that is suitable for compounds that do not withstand heating at high temperatures (Ramaa et al., 2006). The aim is to attract the chemical components based on the principle of mass transfer of substance into the solvent component, where the movement began to occur at the interface layer and then diffuses into the solvent (Sethi et al., 2008). Identification of the structure using a mass spectrometer conducted to determine the compounds contained in the samples analyzed can be seen from the relative abundance of mass fragments of molecules (m/e) of the molecular ion (M +). The more stable a molecular fragment that is formed is, then the fragment will be at a relative abundance of large and have a longer lifespan (Vuorela et al., 2004: Shama et al., 2009).

Gas chromatography and mass spectroscopy analysis of compounds was carried out in methanolic extract as shown in Table 1. The GC-MS chromatogram of the 28 peaks of the compounds detected are shown in Figure 1. Chromatogram GC-MS analysis of the methanol extract

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**Table 1.** Major phytochemical compounds identified in methanolic extract of *Nigella sativa*.

S/N	Phytochemical compound	RT (min)	Formula	Molecular weight	Exact mass	Chemical structure	MS Fragment- ions	Pharmacological actions
1	ß-Pinene	3.173	$C_{10}H_{16}$	136	136.1252		53, 69, 93, 121	<i>Anti</i> -inflammatory
2	D-Glucose ,6-O-α- Dgalactopyranosyl	3.613	$C_{12}H_{22}O_{11}$	342	342.11621	OH OH OH OH	60, 73, 85, 110, 126, 182, 212, 261	Anticoagulant, anti-infl 3matory, psychotomimetic and anticancer activities
3	O-Cymene	3.939	$C_{10}H_{14}$	134	134.10955		51, 58, 65, 77, 91, 103, 119, 134	Anti-oxidant activity
4	DL-Arabinose	4.815	$C_5H_{10}O_5$	150	150.052823	но он	55, 60, 73, 85, 96, 119, 132, 149	Antivirus activity
5	Trans -4-methoxy thujane	4.952	$C_{11}H_{20}O$	168	168.151415		55, 59, 72, 81, 85, 93, 107, 125, 136, 153, 168	Antibacterial and anti-Candida activities

Table 1. Cont'd

6	2-Propyl- tetrahydropyran-3-ol	5.742	$C_8H_{16}O_2$	144	144.115029	OH	55, 73, 101, 116, 144	Anti-allergenic and anti-bacterial
7	Terpinen-4-ol	6.097	$\mathrm{C}_{10}\mathrm{H}_{18}\mathrm{O}$	154	154.135765	но	55, 59, 71, 81, 86, 93, 111, 121, 136, 154	Anti-tumoral activity
8	α- D- Glucopyranoside,Ο-α- D-glucopyranosyl- (1.fwdarw.3)-ß-D-fruc	6.697	$C_{18}H_{32}O_{16}$	504	504.169035	HO OH OH	60, 73, 85, 97, 113, 126, 145, 187	Anticarcinogenic antimutagenic, antineoplastic and <i>anti</i> thrombotic
9	Thymoquinone	7.081	$C_{10}H_{12}O_2$	164	164.08373		53, 68, 77, 93, 96, 108, 121, 136, , 149, 164	Anti-cancer activity
10	2-Isopropylidene-5- methylhex-4-enal	7.378	$C_{10}H_{16}O$	152	152.120115		55, 67, 81, 95, 109, 137, 152	Good antioxidant and <i>anti-</i> inflammatory properties

Table 1. Cont'd

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11	Limonen -6-ol ,pivalate	8.717	$C_{15}H_{24}O_2$	236	236.17763	57, 93, 107, 134, 185, 236	Antioxidant and anti- inflammatory
12	Longifolene	9.158	$C_{15}H_{24}$	204	204.1878	55, 67, 79, 94, 107, 119, 133, 147, 161, 175, 189, 204	Antifeedant, anti tumor, anti inflammatory, antioxidant and antibacterial
13	2-(4- Nitrobutyryl)cyclooctan one	9.839	C <sub>12</sub> H <sub>19</sub> NO <sub>4</sub>	241	241.131408	55, 69, 97, 123, 153, 193, 213, 241	Anti-tumor activity
14	ß-Bisabolene	10.302	$\mathrm{C}_{15}\mathrm{H}_{24}$	204	204.1878	55, 69, 93, 109, 135, 161, 189, 204	Anti-ulcer activity

Table 1. Cont'd

15	1,1-Diphenyl-4- phenylthiobut-3-en-1-ol	10.440	C <sub>22</sub> H <sub>20</sub> OS	332	332.123486	5 OH S	55, 81, 105, 121, 135, 150, 179, 205, 233, 314	Anti-inflammatory properties
16	Phenol, 4-methoxy- 2,3,6-trimethyl	11.069	$C_{10}H_{14}O_2$	166	166.09938	но	53, 67, 77, 83, 91, 107, 123, 135, 151, 166	Antioxidant, anticancer, anti inflammatory and sex hormone activity
17	Pyrrolidin -2-one-3ß- (propanoic acid , methyl ester),5- methylene-4α	12.625	$C_{16}H_{25}NO_5$	311	311.173273	TO NHO NHO	57, 81, 110, 136, 149, 164, 196, 224, 255, 280, 311	Unknown
18	Cholestan-3-ol, 2-methylene-,(3ß,5α)	12.980	$\mathrm{C}_{28}\mathrm{H}_{48}\mathrm{O}$	400	400.370516	HO	69, 81, 95, 149, 175, 227, 260, 315, 400	<i>Anti</i> -inflammatory
19	I-(+)-Ascorbic acid 2,6- dihexadecanoate	15.246	${ m C_{38}H_{68}O_{8}}$	652	652.49142	обоно Обоно	57, 73, 85, 98, 115, 129, 143, 157, 185, 199, 213, 227, 256, 297, 322, 353	Antioxidant, cardio protective, cancer preventive, flavour and anti-infertility

Table 1. Cont'd

20	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	16.636	$C_{19}H_{34}O_2$	294	294.25588		55, 67, 81, 95, 109, 123, 150, 164, 178, 191, 220, 263, 294	Analgesic, <i>anti</i> -inflammatory and ulcerogenic
21	1-Heptatriacotanol	17.867	$C_{37}H_{76}O$	536	536.58962	HD	55, 81, 95, 147, 161, 190, 257	Antioxidant, anticancer, anti inflammatory and to sex hormone activity
22	10,13-Eicosadienoic acid , methyl ester	18.387	$C_{21}H_{38}O_2$	322	322.28718	<b>***********</b>	55, 67, 95, 109, 124, 150, 164, 192, 224, 248, 291, 322	Anti-bacterial and anti-candidal activities
23	E,E,Z-1,3,12- Nonadecatriene-5,14- diol	18.742	$C_{19}H_{34}O_2$	294	294.25588	OH OH	55, 81, 95, 149, 262, 294	Antimicrobial activity
24	9-Octadecenamide ,(Z)	18.960	C <sub>15</sub> H <sub>35</sub> NO	281	281.271864	H <sub>2</sub> N ·	59, 72, 83, 114, 184, 212, 264, 281	Anti-inflammatory activity and antibacterial activity
25	2H- Benzo[f]oxireno[2,3- E]benzofuran-8(9H)- one,9-[[[2-(dimethylar	19.589	C <sub>19</sub> H <sub>32</sub> Ñ <sub>2</sub> O <sub>3</sub>	336	336.241293	NH NH	58, 81, 109, 149, 173, 204, 233, 278, 336	Unknown
26	Phthalic acid , decyl oct-3-yl ester	23.686	$C_{26}H_{42}O_4$	418	418.30831		57, 104, 149, 167, 193, 251, 307	Anti-inflammatory

Table 1. Cont'd

27	1,2- Benzenedicarboxylic acid , bis(8- methylnonyl) ester	24.012	C <sub>28</sub> H <sub>46</sub> O <sub>4</sub>	446	446.33961	9,00	71, 99, 149, 167, 193, 228, 289, 307, 321, 361, 403, 446	Antibacterial activity
28	Stiqmasterol	29.007	$C_{29}H_{48}O$	412	412.370516	HO	55, 69, 83, 133, 213, 255, 300, 351, 369, 412	Biological activities such as anti- diabetic, anti-neoplastic, anti- hypertensive and anti-retroviral

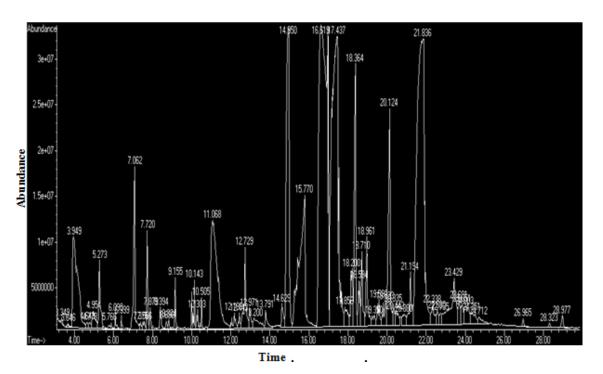
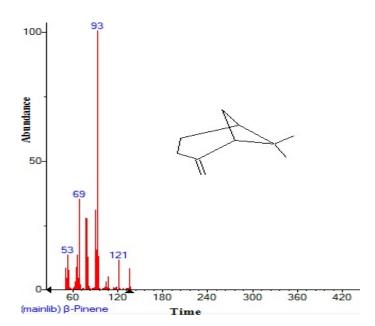


Figure 1. GC-MS chromatogram of methanolic seed extract of Nigella sativa.



**Figure 2.** Structure of ß-Pinene present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.

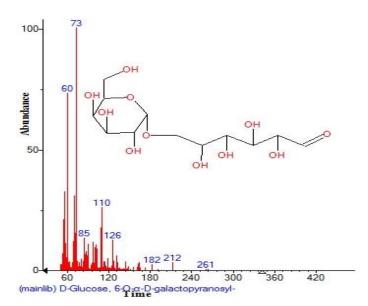
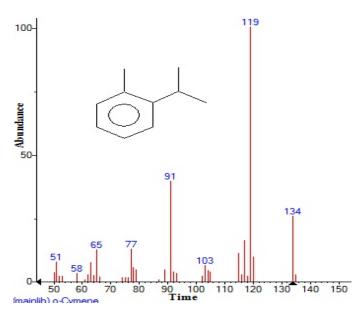


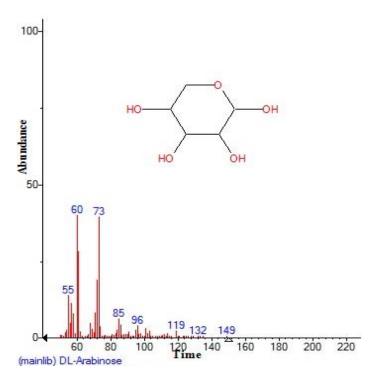
Figure 3. Structure of D-Glucose, 6-O- $\alpha$ -Dgalactopyranosyl present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.

of *N. sativa* showed the presence of 28 major peaks and the components corresponding to the peaks were determined as follows. The first setup peak were determined to be β-Pinene (Figure 2). The second peak showed D-Glucose, 6-O-α-Dgalactopyranosyl (Figure 3). The next peaks was considered to be O-Cymene, DL-Arabinose, Trans -4-methoxy thujane, 2-Propyltetrahydropyran-3-ol, Terpinen-4-ol, α- D-Glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-β-

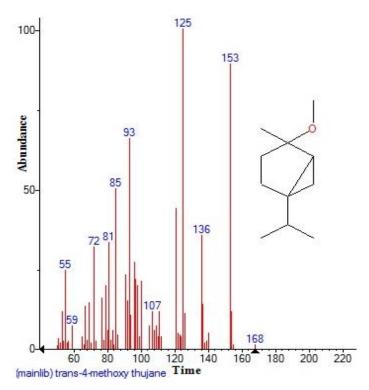


**Figure 4.** Structure of o-Cymene present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.

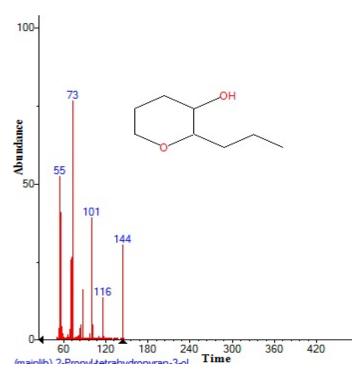
D-fruc, Thymoquinone, 2-Isopropylidene-5-methylhex-4enal, Limonen-6-ol, pivalate, Longifolene, 2-(4-Nitrobutyryl) cyclooctanone. ß-Bisabolene, 1,1-Diphenyl-4phenylthiobut-3-en-1-ol, Phenol. 4-methoxy-2,3,6trimethyl, Pyrrolidin -2-one-3ß-(propanoic acid, methyl ester),5-methylene-4α, Cholestan-3-ol, 2-methylene-,(3ß,5α), I-(+)-Ascorbic acid 2,6-dihexadecanoate. 9.12-Octadecadienoic acid (Z,Z)-,methyl ester, Heptatriacotanol, 10,13-Eicosadienoic acid, methyl ester, E,E,Z-1,3,12-Nonadecatriene-5,14-diol, Octadecenamide,(Z), 2H-Benzo[f]oxireno[2,3-E]benzofuran-8(9H)-one,9-[[[2-(dimethylar, Phthalic acid, decyl oct-3-yl ester, 1,2-Benzenedicarboxylic acid, bis(8methylnonyl) ester and Stigmasterol (Figures 4 to 30). Plants are considered to be one of the natural bases for the production of bioactive compounds, many of which are used to support health and fight against pathological conditions and many of them are marketed as food or herbal medicines (Shama et al., 2009). The usage of herbal medicine has amplified dramatically for various diseases amongst general people over last few years not only because of their easy accessibility without prescription, low cost and appointment to the health care specialists and more with the belief that natural remedies have less lethal effects as compared to synthetic medicines (Ashraf et al., 2011). A qualitative investigation of N. sativa has revealed the presence of sterols, triterpenes, tannins, flavanoids, cardiac glycosides, alkaloids, saponins, volatile oils, volatile glucosinolates and anthraquinones (A1-Yahya, 1986). Qualitative evaluation of the black seed oil via capillary GC-MS technique has enabled the identification of 67 compounds, when classified into various functional



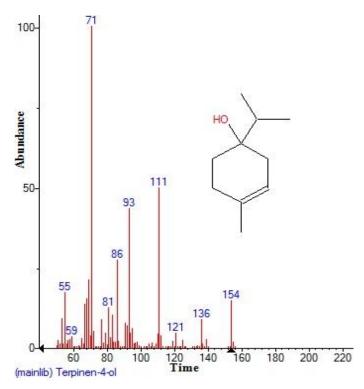
**Figure 5.** Structure of DL-Arabinose present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 6.** Structure of Trans -4-methoxy thujane present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 7.** Structure of 2-Propyl-tetrahydropyran-3-ol present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 8.** Structure of Terpinen-4-ol present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.

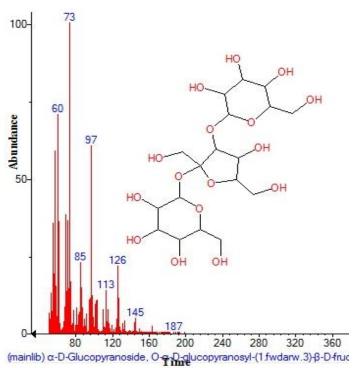
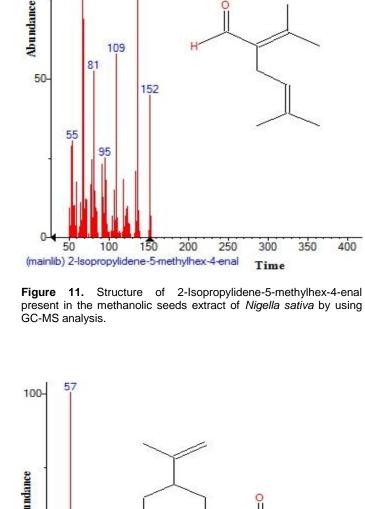


Figure 9. Structure of  $\alpha$ - D-Glucopyranoside,O- $\alpha$ -D-glucopyranosyl-(1.fwdarw.3)-ß-D-fruc present in the methanolic seeds extract of Nigella sativa by using GC-MS analysis.



137

100-

67

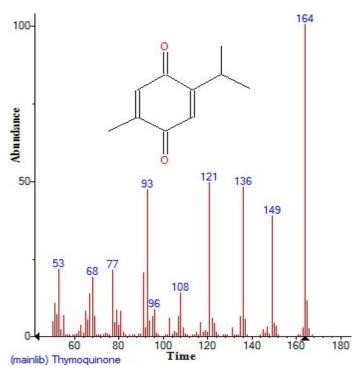
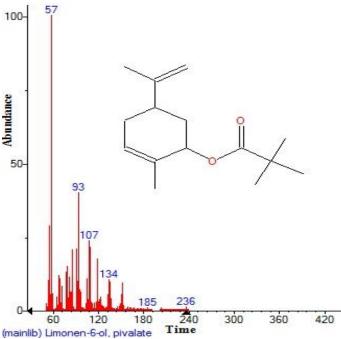


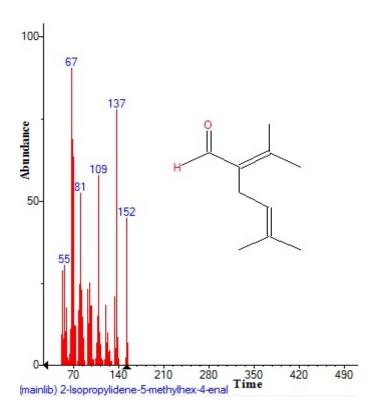
Figure 10. Structure of Thymoquinone present in the methanolic seeds extract of Nigella sativa by using GC-MS analysis.



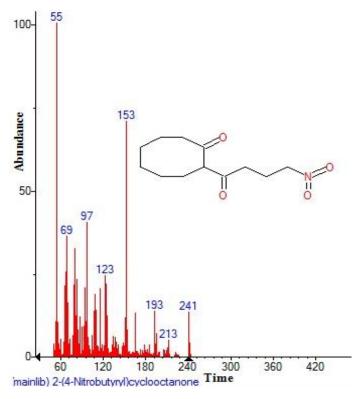
350

400

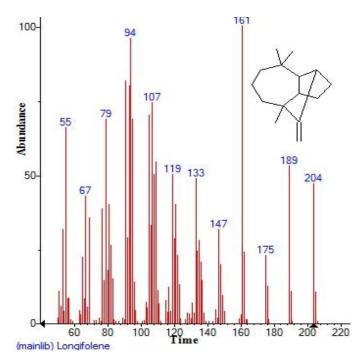
Figure 12. Structure of Limonen -6-ol ,pivalate present in the methanolic seeds extract of Nigella sativa by using GC-MS analysis.



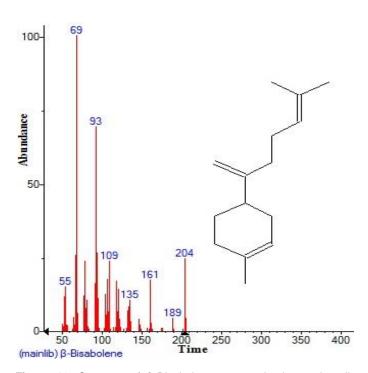
**Figure 13.** Structure of 2-Isopropylidene-5- methylhex-4-enal present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



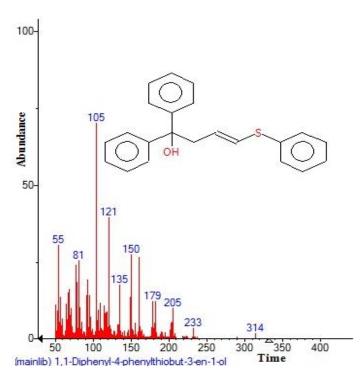
**Figure 15.** Structure of 2-(4-Nitrobutyryl) cyclooctanone present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



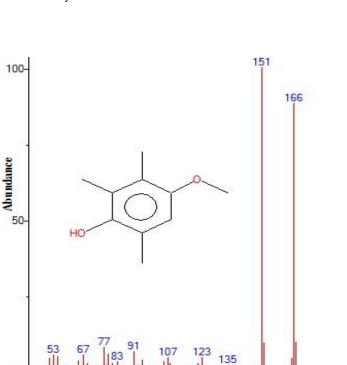
**Figure 14.** Structure of Longifolene present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 16.** Structure of ß-Bisabolene present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 17.** Structure of 1,1-Diphenyl-4-phenylthiobut-3-en-1-ol present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 18.** Structure of Phenol, 4-methoxy-2,3,6-trimethyl present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.

120

Time

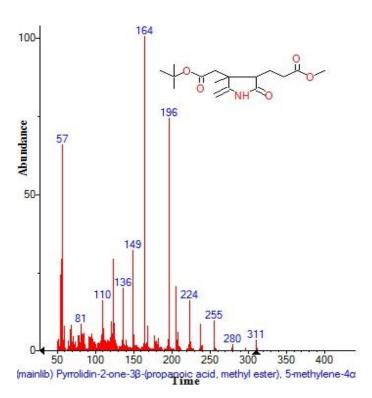
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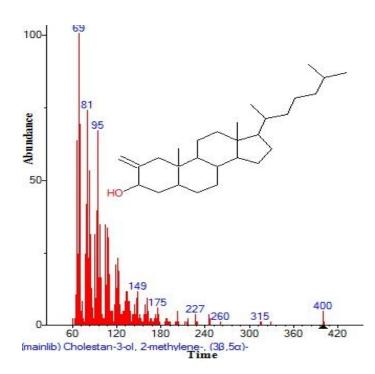
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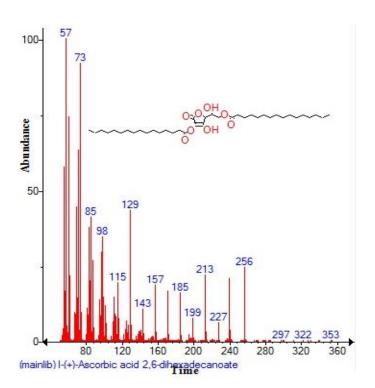
(mainlib) Phenol, 4-methoxy-2,3,6-trimethyl-



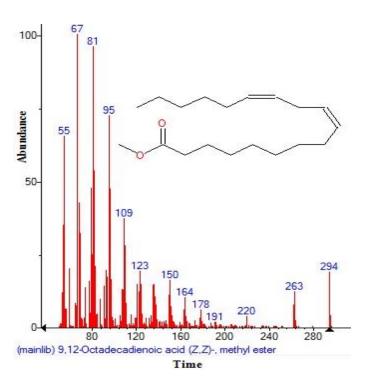
**Figure 19.** Structure of Pyrrolidin -2-one-3 $\beta$ -(propanoic acid , methyl ester),5-methylene-4 $\alpha$  present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



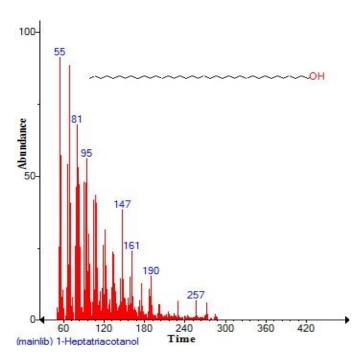
**Figure 20.** Structure of Cholestan-3-ol, 2-methylene-, $(3\mathfrak{G},5\alpha)$  present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



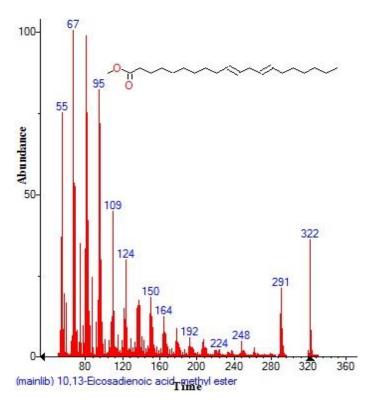
**Figure 21.** Structure of I-(+)-Ascorbic acid 2,6-dihexadecanoate present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



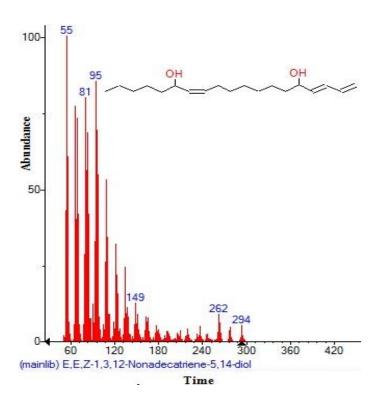
**Figure 22.** Structure of 9,12-Octadecadienoic acid (Z,Z)-, methyl ester present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



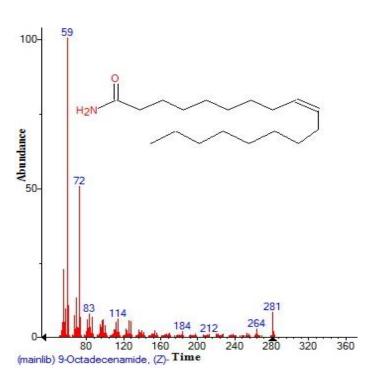
**Figure 23.** Structure of 1-Heptatriacotanol present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



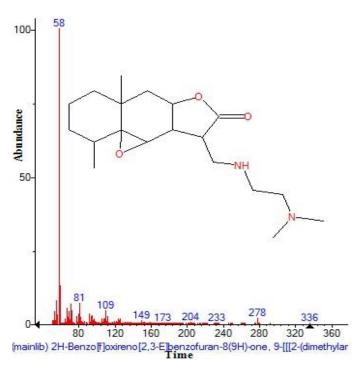
**Figure 24.** Structure of 10,13-Eicosadienoic acid, methyl ester present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



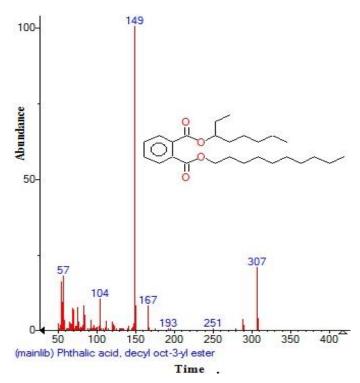
**Figure 25.** Structure of E,E,Z-1,3,12-Nonadecatriene-5,14-diol present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



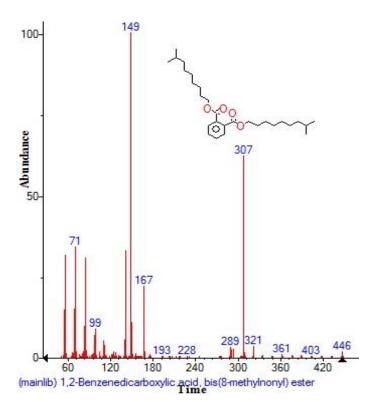
**Figure 26.** Structure of 9-Octadecenamide, (Z) present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



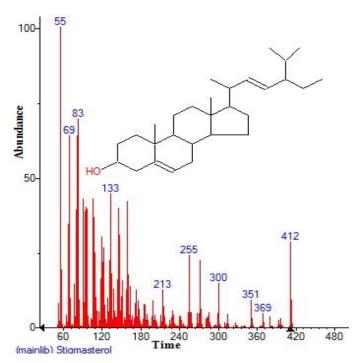
**Figure 27.** Structure of 2H-Benzo[f]oxireno[2,3-E]benzofuran-8(9H)-one,9-[[[2-(dimethylar present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 28.** Structure of Phthalic acid, decyl oct-3-yl ester present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 29.** Structure of 1,2-Benzenedicarboxylic acid , bis(8-methylnonyl) ester present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.



**Figure 30.** Structure of Stiqmasterol present in the methanolic seeds extract of *Nigella sativa* by using GC-MS analysis.

groups corresponding with the following data: monoterpenes (~46%); carbonyl compounds (~25%); phenols (~1.7%); alcohols (~0.9%) and esters (~16%) (Abu-Jadayil et al., 1999).

#### Conclusion

N. sativa seed is a promising source for active ingredients that would be with potential therapeutic modalities in different clinical settings. The efficacy of the active ingredients, however, should be measured by the nature of the disease. It contains chemical constitutions which may be useful for various herbal formulation as anti-inflammatory, analgesic, antipyretic, cardiac tonic and antiasthamatic.

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#### **Conflict of Interests**

The authors have not declared any conflict of interests.

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