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Original article

In-vitro free radical scavenging effect and cytotoxic analysis of *Black Cummins* and Honey formulation

Bharathi Rathi [a], Sandhanasamy Devanesan [b,*], Mohamad S. AlSalhi [b,*], Amirtham J. Ranjith Singh [c]

[a] Department of Biochemistry, Shrimati Indira Gandhi College, Trichy, Tamil Nadu, India
[b] Department of Physics and Astronomy, College of Science, King Saud University, P.O. Box, 2455, Riyadh 11451, Saudi Arabia
[c] Department of Biotechnology, Prathyusha Engineering College, Chennai 600056, India

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ABSTRACT

Background: The antioxidant potential and antiproliferative activity of the extracts of *Nigella sativa* seeds (Black Cummins) and honey formulations are to be explored.
Method: The gas chromatography-mass spectrum (GC–MS) and Thin Layer Chromatography (TLC) fingerprint of *Black Cummins* and Honey formulation revealed alkaloid, saponin, volatile oil, flavonoid, glycosides, sugar, and phenolic compound in the extract. GC–MS profiling of the cold extract of *Nigella sativa* seeds and honey formulation shows peaks for eleven fractions of compounds. Using TLC, the phenolic compounds of *Nigella sativa seeds* and honey formulations were separated.
Results: The current study discovers the cytotoxic effect of black Cummins seeds and honey formulation on human ovarian cancer (PA-1) cell line as assessed by MTT assay. PA-1 cells were inhibited with the increasing concentration of *Nigella sativa* seeds extract and honey formulation.
Conclusion: The study validates the importance of the tested extracts in the treatment of cancer.

1. Introduction

Cancer is an abnormal proliferation of cells that can spread to other areas of the human system. Various mechanisms are widely concerned in the cancer initiation process and changes in the body's normal regulation genes. Cancer is a chief human health crisis that has a considerable impact on the global level. In 2018, approximately 18.1 million new cancer cases were identified worldwide, which are expected to increase to 23.6 million new cases every year by 2030 (Bray et al., 2018). According to Cancer Research in the United Kingdom, 9.6 million patients ended up in death in 2018 among the 17 million cases. If this crisis continues in this way, by 2040, there will be 27.5 million new cancer patients are identified each year (Ashraf, 2020).

Till today there is no precise treatment for several types of cancer with the multidimensional profile. The existing surgical removal and radiation therapy followed by chemotherapy and other therapeutic mechanisms involving DNA-interactive agents, endocrine, and molecular tools leads to severe stress (Nussbaumer et al., 2011). Much-advocated chemotherapy leaves several side effects and other health problems. Hence attention is focused on therapeutic molecules from plants and other natural sources that are safe. The phytochemicals regulate molecular pathways involved in cancer cells' changes and growth (Choudhari et al., 2020).

The ethno botanical derived bioactive compounds are reported to have good remedial properties (Balakrishnan et al., 2020). The bioactive components like alkaloids, glycosides, tannins, flavonoids, and phenolic compounds are reported with novel biomedical applications (Ahmad et al., 2019). Molecular docking showed that the phytochemicals with potential antitumor properties are readily binding with VEGFR and EGFR receptors (Qawoogha and Shahiwala, 2020). The bioactive compounds viz. Curcumin, resveratrol, taxol, vinblastine, vincristine, elliptinium, etoposide, colchicinamide, ipomeanol, lycobetaine, etc. of plants are anticancer agents (Singh et al., 2016).

The Cummins seed of the plant *Nigella sativa* L recommended for disease management by Prophet Muhammad (PBUH) was

* Corresponding authors.
E-mail addresses: dsandhanasamy@ksu.edu.sa (S. Devanesan), malsalhi@ksu.edu.sa (M.S. AlSalhi).
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chosen for the anti-cancerous study (Ali et al., 2018). *N. sativa* is an indigenous herbaceous plant belongs to the Ranunculaceae family that is more commonly known as the fennel flower plant (Shabina Ishtiaq et al., 2013). The bioactive metabolites, Thymoquinone, a-pinene, p-cymene, monoterpenes, etc. are proved medicinal (Rohini et al., 2020). In The Holy Bible, there is also a reference to black Cummins' therapeutic value. The in vitro and in vivo studies proved that the black Cummins seeds have antiviral, antioxidant, immunomodulatory, also, bronchodilators as potential related to SARS-CoV-2 infection suggested it as an adjuvant therapy along with other drugs for COVID-19 patients (Maideen, 2020). The objective of the current study was to determine the antioxidant and antiproliferative activity of the extracts of *Nigella sativa* seeds (Black Cummins) and honey formulations.

2. Materials and methods

2.1. Collection of medicinal plants

The seeds of *Nigella sativa* and honey formulation were collected from an Ayurvedic shop, Lalgudi, Tamil Nadu, and India. The collected *Nigella sativa* seeds and honey formulation were maintained at room temperature in the laboratory.

2.2. Phytochemical analysis

For the phytochemical study, the protocol recommended (Tanaka et al., 1992). The *Nigella sativa* seed extract dissolved in aqueous extract, and Honey was mixed in equal ratio and dissolved in an equal volume of methanol. It was left undisturbed for seven days at room temperature. After the cold extraction, the extract was filtered and kept at 4 °C until use. A specimen was stored for further analysis. After drying the seeds for ten days, the seeds were powdered and used to extract phytochemical compounds.

2.3. Antioxidant properties of Black Cummins and Honey formulation DPPH assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, an antioxidant biomarker, was followed as suggested (Wilkinson et al., 1995). In this assay, 350 μL of 60 μL methanolic DPPH solution and 100 μL of Honey – cummins seeds extract of various concentrations were used. (25–125 mg/ml). After 30 min of reaction, the formulation's absorbance was read at 517 nm against the control blank using a Thermo Scientific S10 spectrophotometer. The effective concentration (EC50), of the formulation needed to decompose 50% of the initial DPPH, was calculated and compared with standard ascorbic acid. The intensity of the color indicates the scavenging role of extract. Phytochemical profiling of the extract using GC–MS analysis was subjected to Gas Chromatography-Mass Spectrometry (GC–MS) analysis. As per the methodology suggested by (Karimi and Jaafar, 2011; AlSalhi et al., 2019).

In the present study, the cytotoxic efficacy of black cumin extract with Honey was tested against human ovarian cancer cell line -PA-1. In the MTT assay, 3 X 104 cells/well was inoculated with the tissue culture medium kept in a 96-well microtitre plate. After incubation overnight, the extract was diluted at five various concentrations and treated along with cell lines for 24 h. Then 20 uL of MTT (5 mg/ml) was added to each microtitre plate (pH 4.7). After the incubation hours, the supernatant was carefully removed, and DMSO was added in all the wells and placed in a shaker for 20 min. The absorbance of the sample was read at 570 nm against blank microtitre plates.

2.4. Thin-layer chromatographic study [TLC]

For TLC studies, the TLC plates were prepared by using the Silica gel 'G'. About 30 gm of silica gel was made to a homogenous suspension with 60 ml distilled water and distributed over the plate. After air dried, the plates were kept in a hot air oven at 110 °C for 30 min and stored. Samples were prepared by diluting the black Cummins seed and honey formulations and applied over TLC plate 2 cm above its bottom and kept in the chromatographic chamber. When the mobile phase moved through the adsorbent phase up to 3/4th of the plate, tests were performed for alkaloids, flavonoids, tannins and phenols, solvent systems.

2.5. Statistical analysis

The experimental calculations were performed using Microsoft 2010 and GraphPad Prism 6.0 software. The mean and standard deviation was significance $P < 0.05$.

3. Results

The phytochemical study of *Nigella sativa* seeds revealed the presence of alkaloid, saponin, quinine, and volatile oil, flavonoids, phenols, tannin, sugar, coumarin, and also the absence of glycosides. The qualitative analysis of Honey revealed the presence of alkaloid, sugar, coumarin, and saponin, whereas flavonoids, phenolic compounds, tannin, glycosides, volatile oil were absent.

The formulation of Black Cummins and Honey, contained alkaloid, saponin, volatile oil, flavonoid, glycosides, sugar, and phenolic compound, whereas coumarin, tannin were absent. (Table 1, Fig. 1, Fig. 2 and Fig. 3).

The presence of chemical compounds were recorded using thin-layer chromatography in figure (Fig. 4) in different solvent system, the bioactive compounds are alkaloids, flavonoids, tannins and phenols and its R_f values recorded in Table 2.

The DPPH free radical scavenging assay accessed the antioxidant potential of *Nigella sativa* seeds and honey formulation indicated the scavenging effect on the DPPH radical about the increase in black concentration Cummins and honey formulations from 200 to1000 mg/ml. The Standard ascorbic acid at the concentration of 1000 mg/ml showed 90% scavenging effect. The IC_{50} value is found to be 20 mg/ml. The extract exhibited more prominent scavenging activity compared to standard, ascorbic acid (Fig. 5).

The result of this GC–MS analysis of Black Cummins and Honey formulation (Fig. 6 and Table 3) indicated the presence of 5-Isopropyl-2-Methylbicyclo[3.1.0]Hex-2-Ene, Benzene, Methyl(1-Methylethyl)-, 4 h-Pyran-4-One, 2,3-Dihydro-3,5-Dihydroxy-6-Methyl-, 1,4-methanoazulene,decahydro-4,8,8-trimethyl-9-methylene,P-Cymene-2,5-Diol, Tetradecanoic Acid, N-Hexadecanoic Acid, Hexadecanoic Acid, Ethyl Ester, 9,12-Octadecadienoic Acid (Z,Z)-, Methyl Ester, 9-Octadecenoic Acid, Methyl Ester, (E)-, Butyl 9,12-Octadecadienoate, Stearic Acid, 2-Hydroxy-1-Methylpropyl Ester, (Z)-18-Octadec-9-Enolide, Methyl 5,13- docosadienoate. The hexadecanoic acid and 9, 12-Octadecadienoic acid is the major bioactive compound found in the Black Cummins and Honey formulation.

The extract formulation reduced the viability of the ovarian cancer PA-1 cells in a concentration-dependent manner for 24 h, and the results are expressed as a % of the control value shown in Fig. 7. Different concentrations (10–70 μg/ml) of black seeds and honey formulation were used in an MTT assay. The black seed and honey formulation extract were more cytotoxic against ovarian cancer PA-1 cell lines. The toxicity percentage increased with an increase of extract concentration, suggestingthat theformula-

Table 1
Phytochemical screening of *Nigella sativa* seeds and honey formulation.

S.NO	Phytochemical name	*Nigella sativa* extract	Honey extract	*Nigella sativa* and honey
1	Flavonoids	–	+	+
2	Phenol	–	–	+
3	Tannins	–	–	–
4	Saponins	+	+	+
5	Coumarins	–	++	+
6	Quinones	–	+++	+++
7	Alkaloids	+++	++	+++
8	Glycosides	+	+	+
9	Sugar	–	+++	++
10	Volatile oils	+++	–	++

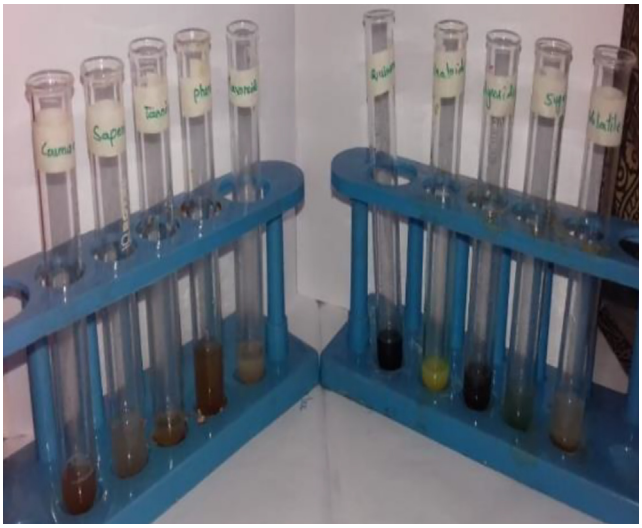

Fig. 1. Phytochemical analysis *Nigella sativa* seeds.

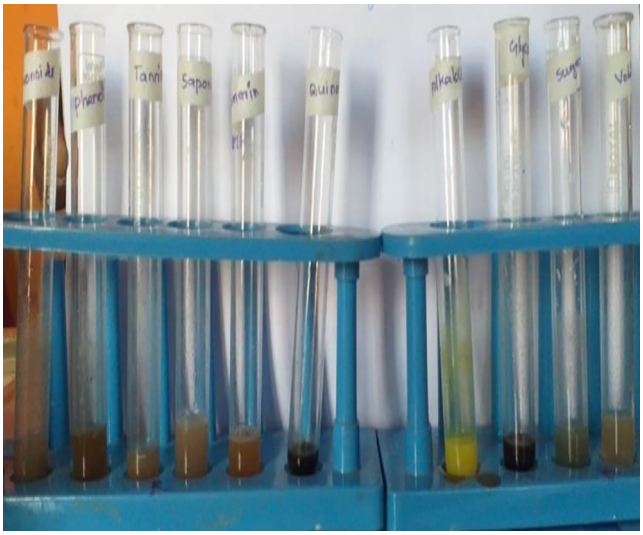

Fig. 3. Phytochemical analysis of *Nigella sativa* seeds and honey formulation.

Fig. 2. Phytochemical analysis of honey.

Fig. 4. Thin layer chromatography of *Nigella sativa* and honey formulation.

tioncould be enormous in the medical field as an anticanceragent. The MTT assayfor the Cytotoxic effect of extract and the ovarian cancer cell line showed that the cell line treated with different concentrations of the extracts (10–70 μg/ml) for 24 h expressed the cell cytotoxicity ratio for PA-1 cells. Data were given as mean ± SD, [error bars indicates statically different experiments compared to control.

Photomicrographs of PA-1 cells treated with black seeds Fig. 8 (a) and the combination of honey extracts and black seeds shows

Table 2
Thin layer chromatography of aqueous extract and its Rf values.

S. No	Extract	No of spot	R_f value
1	Aqueous extract	2	0.31
		2.5	0.39
		1.5	0.23

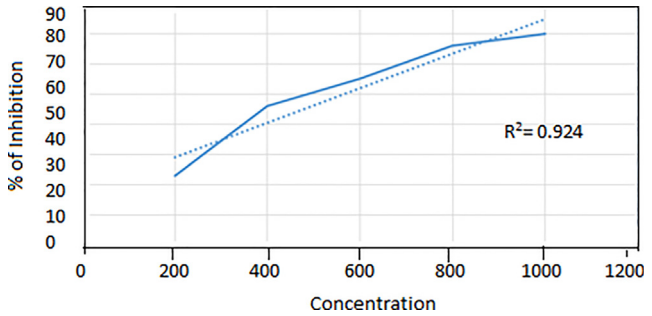

Fig. 5. In-vitro antioxidant activity *Nigella sativa* seeds and honey formulation.

in Fig. 8 (b). The morphological changes in PA-1 cells, such as shrinkage, detachment, membrane blebbing, and distorted shape, are due to extract treatment (40 μg/ml for 24 h) compared with control. Control cells showed normal intact cell morphology, and a light microscope captured their images.

4. Discussion

Scientific evidence confirms that the phytochemicals exhibit a good anticancer activity, and nearly 50% of standard anticancer drugs were plant origin (Newman, 2008; Perumal, et al., 2020). The antioxidant potential of plant bioactive compounds and free radical scavenging reduces cancer cells' growth and inhibits primary and secondary tumors (Majumder et al., 2015). The phytochemicals interfere with molecular targets and signal transduction pathways, including membrane receptors, kinases, downstream tumor-activator or suppressor proteins, transcriptional factors, microRNAs (miRNAs), cyclins, and caspases are affected (Choudhari et al., 2020).

Table 3
GC–MS analysis of *Nigella sativa* seeds and honey formulation.

S. No	Frequency range	Type of bond	Type of group
1	3362.09	N-H stretching	Aliphatic primary amine
2	2128.75	N = C = S	Isothiocyanate
3	1640.59	C = C stretching	Alkene
4	1406.57	C-H bending	Alkane
5	1362.78	S = O stretching	Sulfonate
6	703.96	C = C bending	Alkene
7	602.23	C-Br stretching	Halo compound
8	558.08	C-I stretching	Halo compound

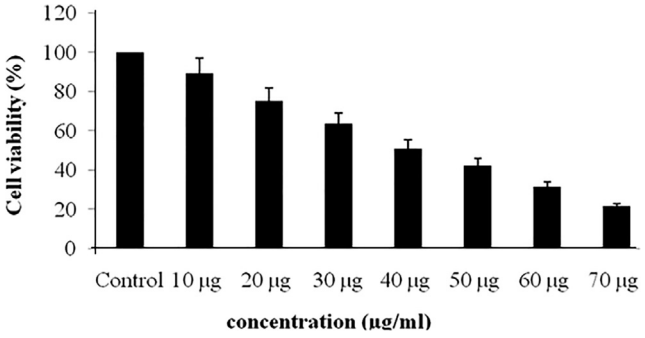

Fig. 7. Cytotoxic effect of extract of ovarian cancer cell line with different 10–70 μg/ml.

The phenolic compounds are anticancer metabolites (Maria et al., 2011) and exhibit many biomedical properties (Mittal et al., 1962). The phenolic contents of Honey are antileukemic in function. The Polyphenols present in Honey are responsible for the antiproliferative activity of various cancer cell lines and tissues (Cook and Samman, 1996). Flavonoids are good antioxidants and

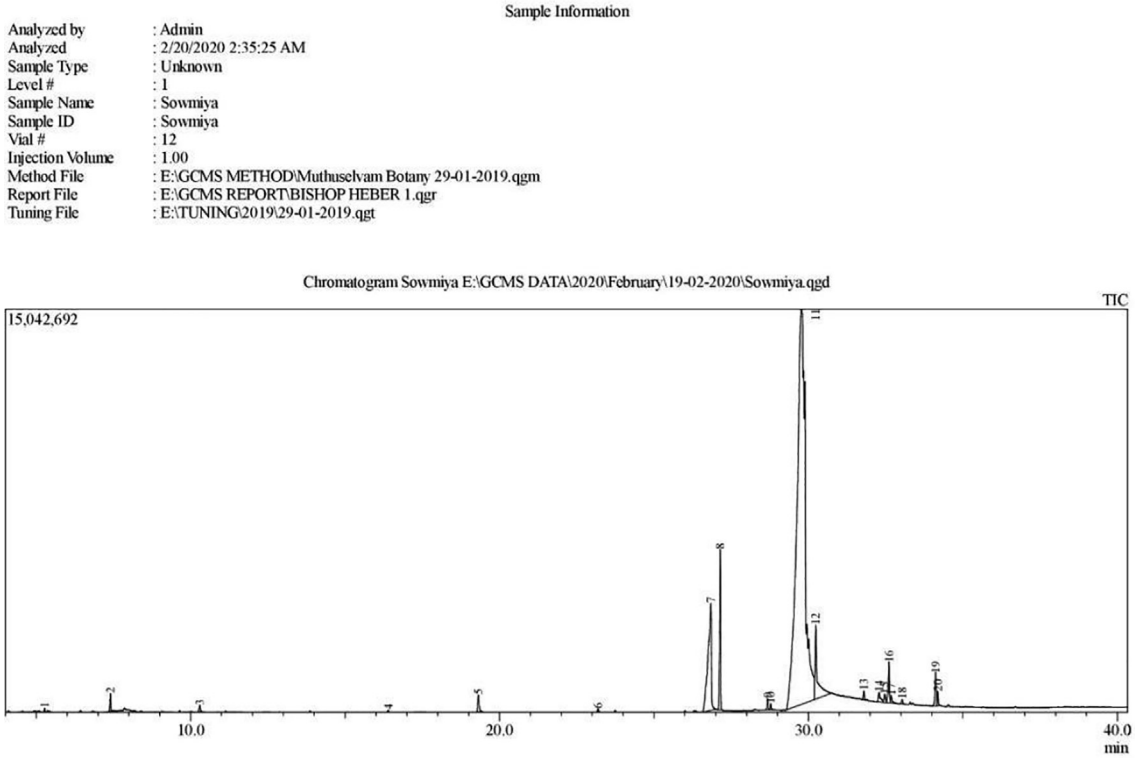

Fig. 6. GC–MS analysis of *Nigella sativa* seeds and honey formulation.

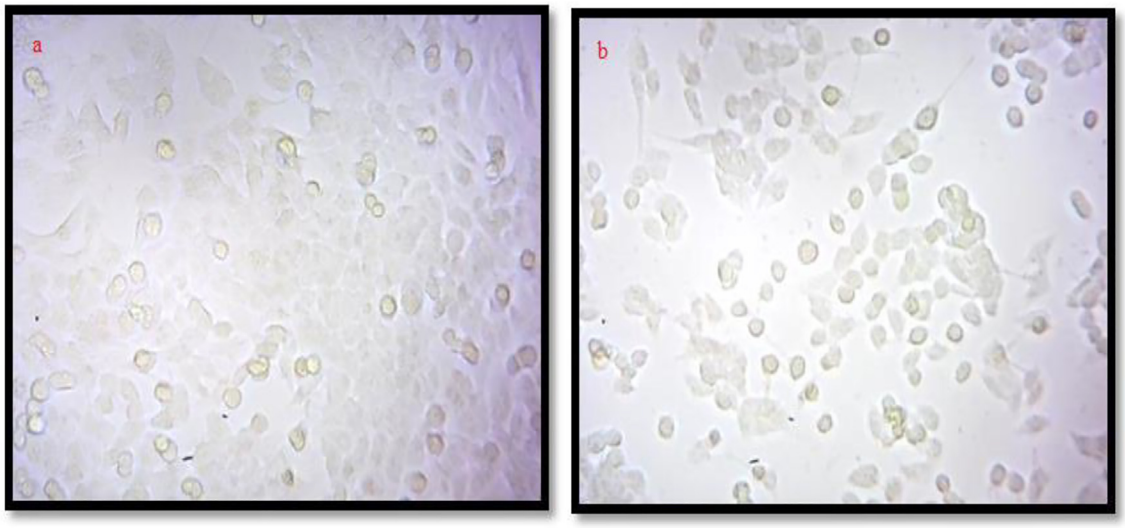

Fig. 8. (a-b) Photomicrographs of PA-1 cells treated with black seeds and a combination of honey and black seeds extracts.

are beneficial in cancer, cardiovascular diseases, and some pathological infections (Jenkins et al., 2002). Flavonoids are reported to reduce carcinogenesis in animal models (Yao et al., 2004).

Alkaloids in plants have a fantastic effect on public health and are powerful painkiller medicines. Also, alkaloids possess anti-inflammatory, anti-asthmatic, and anti-anaphylactic properties with consequences of altered immunological status in vivo (Marimuthu et al., 2012). Saponin present in the phytochemicals allows antibody access to intracellular proteins. It is used in hypercholesterolemia, hyperglycemia, antioxidant, anticancer, anti-inflammatory, and weight loss functions. Also, saponins have anti-tumor, antioxidant, and anti-mutagenic activities and can lower the risk of human cancers by inhibiting cancer cells' proliferation.

DPPH expressing the antioxidant property was because of polyphenolic content, and polyphenols play a vital role in attacking and neutralizing the free radicals (Bourhia et al., 2019; Jeyaprakash et al., 2020). The antioxidant nature of Honey prevents several acute and chronic level disorders such as diabetes and cancer (Hassan et al., 2012). The earlier study reported most of extracted the antioxidant compounds from polar solvents (Dutta and Ray, 2020). The phenolic acids and flavonoids are accountable for Honey's antioxidant and antitumor effect (Erejuwa et al., 2014).

In GC–MS analysis, the reported compounds, Hexadecanoic acid and Octadecanoic acid, have anticancer activity and gastro-protective effect (Farid et al., 2014). The chromatography study have an evidence of honey contains flumethrin residues which are responsible to treat veterinary medicine for parasitic insects (Jamal et al., 2020). Another report showed the honey formulation to inhibit the growth of Entamoeba histolytica and Giardia lamblia trophozoites (Mohammed et al., 2019). The numerous biological benefits were recorded from the *Nigella sativa* seeds which is accrued from quinone family. The aromatic compounds are mostly effective for treatment in diverse human cancer cell lines (Penecilla and Magno, 2011), So that the present study suggests that the mixed formulation has several pharmacological activities. Further studies are needed to find out the beneficial effects of the formulation.

5. Conclusions

The novel bioactive compounds present in the seeds of *Nigella sativa and Honey* reduced tumor cell proliferation in a dose-dependent manner without affecting normal cells. Hence the extracts of *Nigella sativa* seedsand Honeyformulation could be a new therapeutic source in antitumor therapies. Further studies can be done on this extract to obtain more valuable data for formulating significant anticancer bioactive compounds, potential mode of action, and take the research in advance for further exploration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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