IJPSR (2019), Volume 10, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 10 May 2018; received in revised form, 25 July 2018; accepted, 21 November 2018; published 01 January 2019

ACETYLCHOLINESTERASE AND GROWTH INHIBITORY EFFECTS - VARIOUS GRADES OF *N. SATIVA* OILS

R. Kannan *, Sibi P. Ittiyavirah and Jyoti Harindran

Department of Pharmaceutical Sciences, Centre for Professional and Advanced Studies, Kottayam - 686631, Kerala, India.

Keywords:

Acetylcholinesterase, Sulforhodamine B assay, Black cumin oil, Neuroprotective

Correspondence to Author:
R. Kannan

Department of Pharmaceutical Sciences, Centre for Professional and Advanced Studies, Kottayam - 686631, Kerala, India.

E-mail: kannanramalingam.g@gmail.com

ABSTRACT: Neuroprotection is utmost necessary due to the devastating neuronal injuries, including neurodegenerative disorders. *N. sativa* L. is called the Miracle herb due to its wide usage in traditional systems of medicine, especially Arabic. This plant has been studied for its therapeutic potential and found to possess a wide spectrum of activities, including anticancer and antioxidant properties. The present work is designed to investigate the acetylcholinesterase inhibitory and cytotoxic potential of various grades of black cumin oil for its neuroprotective activity. Thymoquinone is the most pharmacologically active ingredient found in black cumin oil. The various grades of black cumin containing 5%, 2% and 0.6% thymoquinone at different dose levels is used throughout this work. Acetylcholinesterase inhibitory is performed by using Ellman's method, and the cytotoxic potential is evaluated by using Sulforhodamine B assay employing IMR-32, U373-MG, and SK-N-SH cell lines. Black cumin oil containing 5% thymoquinone showed a dose-dependent increase in the acetylcholinesterase inhibition (64.47% inhibition at the dose of 20 µg/ml) which is similar to that of the standard acetylcholinesterase inhibitor donepezil (65.03% inhibition at the dose of 10 µg/ml). The cytotoxic potential evaluated by SRB assay indicated the black cumin oil containing 5% thymoquinone exhibiting the comparable growth inhibition with that of Adriamycin. Whereas, other grades of black cumin oil showed limited or no activity. It is concluded that *N. sativa* oil exhibits a neuroprotective activity in both the assays in a dose-dependent manner which indicates the variable effects of the concentration of thymoquinone present in the black cumin oil.

INTRODUCTION: Neuroprotection refers to the strategies and relative mechanisms which can defend the central nervous system (CNS) against neuronal injury due to both acute (*e.g.*, stroke or trauma) and chronic neurodegenerative disorders (*e.g.* Alzheimer's disease and Parkinson's disease) [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(1).245-50

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(1).245-50

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by impairment of memory and cognitive function. Initially, mild cognitive impairment and deficits in short-term and spatial memory appear, but the symptoms become more severe with disease progression [2]. In traditional practices of medicine, plants have been used to enhance cognitive function and to alleviate other symptoms associated with AD [3].

Nigella sativa L. (Black cumin) is an annual herb belonging to the Ranunculaceae family. It is naturally distributed in countries bordering the

Mediterranean Sea and India. The black cumin seeds and the oil are being widely used over centuries for the treatment of various ailments throughout the world. It is one of the important drugs in traditional Indian systems like Ayurveda and Unani. Many studies on *N. sativa* suggest that its biological activity is attributed specifically to the components in the essential oil. So far, many active compounds have been identified and isolated from black cumin seeds. The major active constituents present in black cumin seeds are thymoquinone (30-48%), p-cymene (7-15%), carvacrol (6-12%), 4-terpineol (2-7%), t-anethole (1-4%), sesquiterpene longifene (1-8%) to name a few [4, 5].

Thymoquinone (TQ) which is chemically called as 2-isopropyl -5- methyl-1, 4- benzoquinone is the most pharmacologically active ingredient found in black cumin oil. It was reported that thymoquinone possessed various bioactivities, including anti-cancer and anti-oxidant properties [6]. Acute and chronic toxicity studies of black cumin oil and thymoquinone on laboratory animals were reported and stated to be safe, particularly when administered orally. In black cumin oil, the amount of TQ present is about 0.6%. In this study, we investigated the various grades of black cumin oil for its Acetylcholinesterase activity and also its cytotoxic potential by employing *in-vitro* methods.

MATERIALS AND METHODS:

Materials: Black cumin oils containing 2% TQ (TQT), 5% TQ (TQF) and normal black cumin oil containing 0.6% TQ (TQN) was obtained from Akay Flavours & Aromatics Pvt. Ltd., Kerala, India. Ellman's reagent (DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid) (D8130), Acetylthiocholine iodide (01480), Acetylcholinesterase Type VI-S, (C3389) from electric eel, 200-1,000 units/mg protein were purchased from Sigma-Aldrich, Inc.

Methods:

Determination of Acetylcholinesterase Inhibitory Activity: Acetylcholinesterase (AChE) activity was determined according to the method of Ellman *et al*., (1961). Inhibition of AChE activity was measured using a UV-Spectrophotometer based on Ellman's method. The enzyme hydrolyzes the substrate Acetylthiocholine Iodide to thiocholine and acetic acid. Thiocholine is allowed to react with DTNB, and this reaction resulted in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity. 500 µl of DTNB 3 mM, 100 µl of Acetylthiocholine iodide (AChI) 15 mM, 275 µl of Tris–HCl buffer 50 mM, pH 8 and 100 µl of each sample was dissolved in ethanol, water or DMSO, respectively, and were added to a 1 ml cuvette. This cuvette was used as a blank. In the reaction cuvette, 25 µl of buffer was replaced by the same volume of an enzyme solution 0.28 Uml^{-1}. The reaction was monitored for 5 min at 412 nm. The values were taken in triplicate [8, 9].

The percentage inhibition for each test solution was then calculated using the following equation:

Inhibition (%) = 1- (Absorbance of the sample /Absorbance of the blank) × 100

Evaluation of Cytotoxic Potential by Sulforhodamine B Assay: The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h before addition of experimental drugs.

Experimental drugs were initially solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1mg/ml using water and stored frozen before use. At the time of drug addition, an aliquot of frozen concentrates (1 mg/ml) was thawed and diluted to 100 g/ml, 200 g/ml, 400 g/ml and 800 g/ml with complete medium containing test articles. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of the medium, resulting in the required final drug concentrations, i.*e.,* 10 g/ml, 20 g/ml, 40 g/ml, 80 g/ml.

After compound addition, plates were incubated under standard conditions for 48 h, and the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C.

The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, the unbound dye was recovered, and the residual dyes were removed by washing five times with 1% acetic acid. The plates were air dried. The bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed in the ratio of average absorbance of the test well to the mean absorbance of the control wells × 100.

Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels.

Percentage growth inhibition was calculated as:

$$[Ti/C] \times 100\%$$

A growth curve was constructed using percentage control growth versus drug concentration using the growth curve the concentration of drug causing 50% inhibition of cell growth (GI_{50}) was calculated. It was stated that the GI_{50} value of 10 or less than 10^{-6} is considered to demonstrate activity [10, 11].

RESULTS AND DISCUSSION: Acetylcholine, a neurotransmitter is essential for processing memory and learning. Pharmacological inhibitors of AChE are important in controlling diseases that involve impaired acetylcholine-mediated neurotransmission. For example, Alzheimer's disease (AD) involves selective loss of cholinergic neurons in the brain. In myasthenia gravis, auto-antibodies reduce the number of nicotinic acetylcholine receptors at the neuromuscular junction. AChE inhibition increases the synaptic concentration of acetylcholine and allows a higher occupancy rate and longer duration at its receptor – donepezil hydrochloride; an AChE inhibitor is used for the treatment of AD and other neurological disorders [12].

In our study AChE inhibitor activity of black cumin oil is estimated by using Ellman's method. The black cumin oils at the concentrations of 5%, 2% and normal black cumin oil at different doses were evaluated employing Donepezil (DPL) as the standard.

Jukic *et al*., 2007 studied the *in-vitro* AChE inhibitory potential on thymoquinone and their derivatives and reported the repressive potential of them as thymohydroquinone > carvacrol > thymoquinone > total essential oil > thymol > linalool. In the present work, we confirmed the AChE inhibitory activity of thymoquinone rich black cumin oil, which produced dose-dependent activity. The AChE inhibitory potential decreased in the following order: DPL > TQF > TQT > TQN. The inhibitory effect of the 20µg/ml concentration of TQF corresponds to the 10µg/ml concentration of DPL. Inhibitory activity of TQT was very weak and TQN even produced negative results. **Fig. 1** shows the AChE inhibitory activity of black cumin oil samples and the standard.

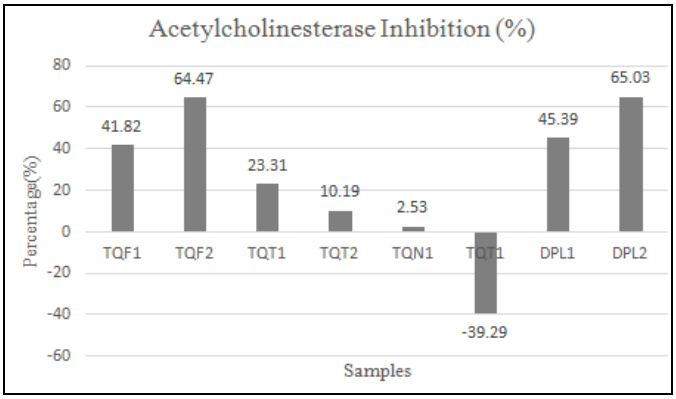

FIG. 1: AChE INHIBITORY ACTIVITY OF DIFFERENT GRADES OF BLACK CUMIN OIL USING ELLMAN'S METHOD. Where TQF1 - Black cumin oil containing 5% TQ (10µg/ml), TQF2 - Black cumin oil containing 5% TQ (20µg/ml), TQT1 - Black cumin oil containing 2% TQ (10µg/ml), TQT2 - Black cumin oil containing 2% TQ (20µg/ml), TQN1 - Black cumin oil containing 0.6% TQ (10µg/ml), TQN2 - Black cumin oil containing 0.6% TQ (20µg/ml), DPL1 – Donepezil (5µg/ml), DPL2 – Donepezil (10µg/ml).

Certain data suggest that cancer survivors have a decreased risk of Alzheimer's disease. A link between cancer and neurodegeneration is plausible as they share several genes and biological pathways, including inappropriate activation and deregulation of the cell cycle. Signaling along these pathways results in opposite endpoints: in the case

of cancer, uncontrolled cell proliferation, and in the case of neurodegeneration, apoptotic cell death. Proteins such as p53, a major regulator of apoptosis, and Pin1, which has a dual role in cell cycle control and protein folding, play a key part in the pathophysiology of both Alzheimer's disease and cancer [14].

Sulforhodamine B assay provides a rapid and sensitive method for measuring the drug-induced effects in both attached and suspension cultures. Sulforhodamine B is a bright pink aminoxanthone dye with two sulfonic groups, which bind to protein basic amino acid residues under mildly acidic conditions and the color development in the assay is rapid, stable and visible. The optical density of sulforhodamine B assay can be measured over a broad range of visible wavelengths in either a spectrophotometer or a 96-well plate reader. The major advantage of this assay over others is that this dye would not stain cell debris. Therefore, the sensitivity of sulforhodamine B assay is not affected by the presence of cell debris. So, it has been widely used for the cell growth studies [11, 15].

Three different cell lines IMR-32; U373-MG and SK-N-SH were used here in this study to evaluate the efficacy of the drug samples which contain three various concentrations of thymoquinone. The IMR-32 cell lines are a continuous hyperdiploid human neuroblastoma cell line, and this cell line is proved to be an excellent source for the isolation of human neuronal nAChR subunit cDNAs. The acetylcholine neurotransmitters play a vital role in both memory and behavior. Furthermore, it is also stated that wild-type human neuroblastoma IMR-32 cells can secrete long amyloid -protein [16, 17]. Amyloid beta acts as a neurotoxin when gets accumulated in the brain whereas the other cell line U373-MG which is human astrocytoma cell lines.

This cell line is proved to provoke inflammatory markers like IL-6 when triggered with certain inducers, and it is as well found that serotonin receptors can be expressed/ unexpressed to modify the release of IL-6. [18] SK-N-SH is a continuously cultured human neuroblastoma cell line, which is well known for studies related to neurodegenerative diseases. It is also proved that these cell lines are capable of synthesizing multiple neurotransmitters which includes dopamine, norepinephrine, acetylcholine, GABA, *etc*. The black cumin oil with different concentrations of thymoquinone; TQT, TQN, and TQF were used. Adriamycin (ADR) is used as a positive control whereas DPL is used as a standard. All the drug samples were dissolved in DMSO for the assay. The drug samples at the concentrations of 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml were used. The assay is done in triplicate, and the average values were used to determine the percentage control growth.

From the growth curve of IMR-32 cell lines, the GI_{50} of TQF was found to be 4.3, which showed maximum activity among all the drug samples followed by TQN at 24.1 and TQT at 30.9, which prove the dose-dependent activity of TQ in black cumin oil. The DPL is found to produce no activity whereas the positive control produced the GI_{50} value of less than 10. The growth curve of the IMR-32 cell line was shown in **Fig. 2A** and GI_{50} values in **Table 1**. When compared to neuroblastoma cell lines, the activity of black cumin oil is found to produce less action on glioblastoma cell line U373-MG. The GI_{50} value of TQF was 18.3 whereas of TQN, and TQT was 38.1 and 43.3 respectively. Here too donepezil has not produced any significant activity whereas Adriamycin produced GI_{50} of less than 10. The growth curve of the U373-MG cell line was shown in **Fig. 2B** and GI_{50} values in **Table 1**.

In SK-N-SH cell line, the drug samples TQF, TQT, and ADR were found to produce activity whereas TQN had shown the GI_{50} value of 35.4 and Donepezil with greater than 80. The growth curve of the SK-N-SH cell line was shown in **Fig. 2C** and GI_{50} values in **Table 1**. The SRB assay in the present study implicit that TQF produces activity, especially on neuronal cells (IMR-32 & SK-N-SH) than glial cells (U373-MG). Whereas TQT produced activity only on SK-N-SH cell line, and TQN produced limited or no activity on all the three cell lines, which replicates the action of black cumin oil based on TQ concentration.

CONCLUSION: In summary, DPL, the standard drug, and TQF exhibited a dose-dependent increase in AChE inhibitory activity. While TQT and TQN exhibited a dose-dependent decrease in AChE inhibition. This might be due to the variation in the active content namely TQ present in the oil of *N.*

sativa. These results are also correlating with GI_{50} values of various grades of black cumin oil as observed from the growth curve. Adriamycin is producing a consistent inhibition in all three cell lines. TQF is also producing a comparable effect while other samples TQT, TQN, and DPL failed to produce such growth inhibition. This work confirms the effect of various grades of black cumin oil for its neuroprotective activity. This study gives an insight into a thorough safety pharmacological studies for the oil of *N. sativa*.

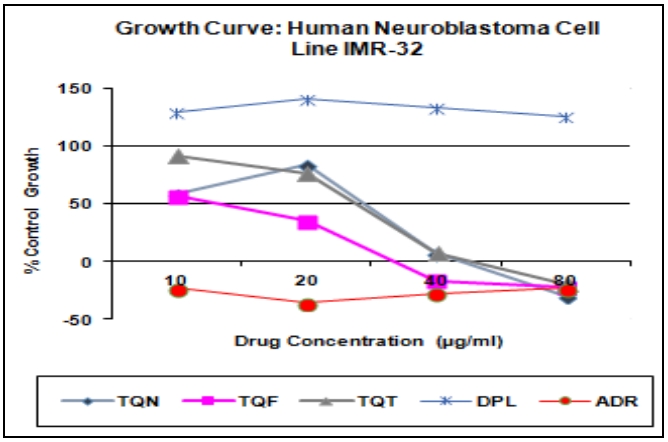

FIG. 2A: GROWTH CURVE: HUMAN NEUROBLASTOMA CELL LINE IMR-32 USING SRB ASSAY. Where TQF- Black cumin oil containing 5% TQ, TQT - Black cumin oil containing 2%, TQN - Black cumin oil containing 0.6% TQ, DPL – Donepezil, ADR - Adriamycin

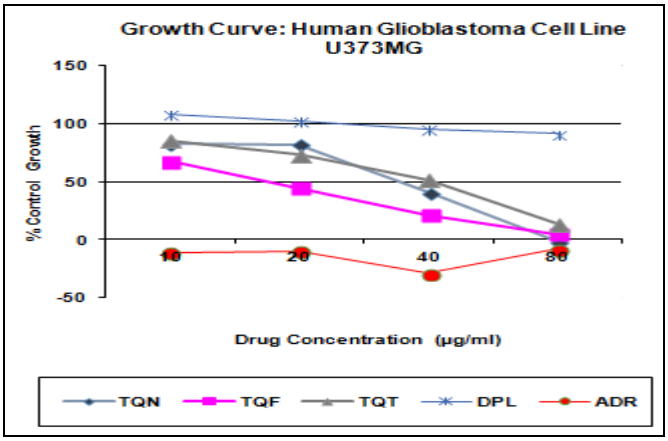

FIG. 2B: GROWTH CURVE: HUMAN GLIOBLASTOMA CELL LINE U373MG USING SRB ASSAY. Where TQF- Black cumin oil containing 5% TQ, TQT - Black cumin oil containing 2%, TQN - Black cumin oil containing 0.6% TQ, DPL - Donepezil, ADR - Adriamycin

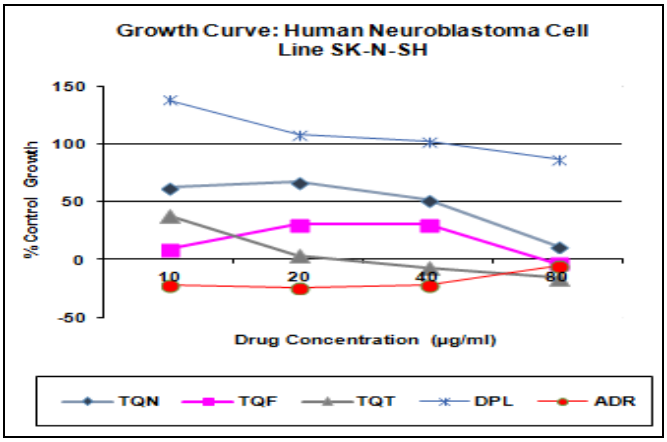

FIG. 2C: GROWTH CURVE: HUMAN NEUROBLASTOMA CELL LINE SK-N-SH USING SRB ASSAY. Where TQF- Black cumin oil containing 5% TQ, TQT - Black cumin oil containing 2%, TQN - Black cumin oil containing 0.6% TQ, DPL - Donepezil, ADR - Adriamycin

TABLE 1: GI_{50} VALUES OF DIFFERENT GRADES OF BLACK CUMIN OIL ALONGSIDE WITH DONEPEZIL AND ADRIAMYCIN

Samples	Drug concentrations (µg/ml) calculated from the graph		
	IMR-32	U373MG	SK-S-NH
TQN	24.1	38.1	35.4
TQF	4.3	18.3	<10
TQT	30.9	43.3	<10
DPL	NE	NE	>80
ADR	<10	<10	<10

ACKNOWLEDGEMENT: We express our gratitude to Akay Flavours & Aromatics Pvt. Ltd., Kerala, India for the supply of different grades of standardized black cumin oil.

In-vitro SRB assay for anti-cancer activity evaluation of drugs was done at Anti-Cancer Drug screening facility (ACDSF) at ACTREC, Tata Memorial Centre, Navi Mumbai.

CONFLICT OF INTEREST: The authors declare no conflict of interest

REFERENCES:

1. Kumar GP and Khanum F: Neuroprotective potential of phytochemicals. Pharmacogn Rev 2012; 6(12): 81-90.
2. Merlo S, Spampinato S, Canonico PL, Copani A and Sortino MA: Alzheimer's disease: Brain expression of a

metabolic disorder? Trends Endocrinol Metab 2010; 21(9): 537-44.
3. Howes MJR and Houghton PJ: Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacol Biochem Behav 2003; 75(3): 513-27.
4. Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK and Siddique NA: A review on the therapeutic potential of Nigella sativa: A miracle herb. Asian Pac J Trop Biomed [Internet]. 2013; 3(5): 337-52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23646296
5. Leong XF, Mustafa RM and Jaarin K: *Nigella sativa* and its protective role in oxidative stress and hypertension. Evidence-Based Complement Altern Med [Internet]. 2013 2013; 1-9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23533459
6. Salim LZA, Mohan S, Othman R, Abdelwahab SI, Kamalidehghan B and Sheikh BY: Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro. Molecules 2013; 18(9): 11219-40.
7. Nickavarm B, Mojab F, Javidnia K and Amoli MA: Chemical composition of the fixed and volatile oils of Nigella sativa L. from Iran. 2003; 58(9-10): 629-631tle. Z Naturforsch C 2003; 58(9-10): 629-31.
8. Adewusi EA and Steenkamp V: *In-vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Southern Africa. Asian Pac J Trop Med [Internet]. 2011; 4(10): 829-35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22014742
9. Ferreira A, Proença C, Serralheiro MLM and Araújo MEM: The *in-vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J Ethnopharmacol 2006; 108(1): 31-7.
10. Orellana E and Kasinski A: Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. BIO-PROTOCOL 2016; 6(21): e1984. Available from: http://www.bio-protocol.org/e1984
11. Vichai V and Kirtikara K: Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 2006; 1(3): 1112-6.
12. Soreq H: Acetylcholinesterase - new roles for an old actor. Nat Rev Neurosci 2001; 2(4): 294-302.
13. Jukic M, Politeo O, Maksimovic M, Milos M and Milos M: *In-vitro* acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phyther Res 2007; 21(3): 259-61.
14. Driver JA, Beiser A, Au R, Kreger BE, Splansky GL and Kurth T: Inverse association between cancer and Alzheimer's disease: results from the Framingham Heart Study. BMJ 2012; 344(12): 1442.
15. Voigt W: Sulforhodamine B Assay and Chemosensitivity. Chemosensitivity [Internet]. 2005; 110: 039-48. Available from:http://link.springer.com/10.1385/1-59259-869-2:039.
16. Asami-Odaka A, Ishibashi Y, Kikuchi T, Kitada C and Suzuki N: Long amyloid beta-protein secreted from wild-type human neuroblastoma IMR-32 cells. Biochemistry 1995; 34(32): 10272-8.
17. Kormelink GPJ and Luyten WH: Cloning and sequence of full-length cDNAs encoding the human neuronal nicotinic acetylcholine receptor (nAChR) subunits beta3 and beta4 and expression of seven nAChR subunits in the human neuroblastoma cell line SH-SY5Y and/or IMR-32. FEBS Lett 1997; 400(3): 309-14.
18. Lieb K, Biersack L, Waschbisch A, Orlikowski S, Akundi RS and Candelario-Jalil E: Serotonin *via* 5-HT7 receptors activates p38 mitogen-activated protein kinase and protein kinase C ε resulting in interleukin-6 synthesis in human U373 MG astrocytoma cells. J Neurochem 2005; 93(3): 549-59.

How to cite this article:
Kannan R, Ittiyavirah SP and Harindran J: Acetylcholinesterase and growth inhibitory effects - various grades of *N. sativa* oils. Int J Pharm Sci & Res 2019; 10(1): 245-50. doi: 10.13040/IJPSR.0975-8232.10(1).245-50.