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Anticancer properties of black seed (*Nigella sativa* L.) seedling and/or callus extract against human breast adenocarcinoma and colon cancer cell lines

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Callus was initiated from leaf explants of *Nigella sativa* on MS media supplemented with different plant growth regulator (PGRs) after five weeks. To improve callus growth, fresh callus was subcultured on MS media supplemented with different concentrations of TDZ [(Thidiazuron), 1-Phenyl-3-(1,2,3,-thiadiazol-5-yl) urea], BAP (6-benzylaminopurine), and NAA (α-Naphthalene Acetic Acid) separately or in combinations. All callus treatments were subcultured for three successive subcultures every five weeks in the dark, 10 and 20 µmol m^{-2} s^{-1} light intensity condition. The maximum callus biomass (fresh and dry) was obtained with 2 mg L^{-1} TDZ, and it seemed that the increase in light intensity synergized the effect of TDZ, but the rate of growth declined with repeated subculturing. The potential of callus to synthesize thymol could be proved by high performance liquid chromatography (HPLC) analysis. Data revealed that thymol content in callus obtained on MS media supplemented with 2 mg L^{-1} TDZ reached up to 118.73 µg g^{-1} dry weight. In an experiment to investigate the cytotoxic activity of seedling and callus methanol extract of *N. sativa* using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, the seedling extract gave higher cytotoxic activity against human breast adenocarcinoma (MCF-7), while the callus extract exhibited promising cytotoxic properties against human colon cancer (Caco-2) cell lines. The data presented in this study may represent additional evidence of the promising role that *in vitro* plant cell cultures could play in the production of valuable phytopharmaceutics.

Keywords: callus culture, thymol, Caco-2, MCF-7, plant growth regulators, toxicity

INTRODUCTION

Black seed (*Nigella sativa* L.) belongs to the family "Ranunculaceae", also known as black cumin and it is widely distributed in different regions of the world. Black seed is known to have many therapeutic effects which are attributed to its many active constituents including antioxidative, flavoring, thymol, thymoquinone, dithymoquinone, and carvacrol (Houghton et al. 1995; Meena et al. 2017). Also, black seed is rich in vitamins, phospholipids, fatty acids, ascorbic acid, nigellimine-N-oxide, nigellicine, nigellidine, and alpha-hederin (Khan 1999; Nickavar et al. 2003; Randhawa and Alghamdi 2011). The presence of such valuable constituents seems to be the reason behind the many therapeutic properties of *N. sativa* seeds like the antioxidative thymoquinone protective effects against carbon tetrachloride induced hepatotoxicity (Nagi et al. 1999). For all of that, black seeds have a very common use in Arabic, Southeast Asia and Middle East traditional medicine for treatment of

fever, common cold, headache, asthma, rheumatic disease, microbial infections, cancer and expelling of intestinal worms and anti-inflammatory (Alemi et al. 2013; Randhawa and Alghamdi 2011).

Plant tissue culture techniques have become so promising as an alternative method for production of the drug-like components from the medicinal plants (Sauerwein et al., 1992) and had been extensively studied to improve the production of phytochemicals (Zhao et al., 2005) *in vitro*. Positive results has been obtained from trials to induce callus formation from different explants of *N. sativa* using a variety of plant growth regulators (PGRs) with the purpose of studying the expression of secondary phytopharmaceutics and specific constituents like thymol in callus cultures (Chaudhry et al., 2014; ElNour et al., 2015; Ibrahim et al., 2015). Thymol is one of the powerful phenolic antioxidants present in the essential oil of *N. sativa* seeds and is known to play an essential role in inhibition of cancer cells beside its antibacterial action against oral bacteria (Chaudhry et al., 2014). Furthermore, the extract of *N. sativa* has been shown to have activity against some Gram-positive and Gram-negative bacteria (Hanafy and Hatem 1991). Yasni et al., (2009) found out that the ethanol extract of black cumin (*N. sativa*) markedly inhibited the growth of many pathogenic and food spoilage bacteria.

The anticancer properties *of N. sativa* were documented by Ibn-Sina more than 1000 years ago (Al-Jishi 2000). *N. sativa* seeds were shown to have cytotoxic effects against different groups of cancer cell lines (Swamy and Tan 2000), restriction of soft tissue sarcomas persuaded in albino mice by 20-methylcholanthrene (Salomi et al., 1991), deactivation of MCF-7 breast cancer cells (Farah and Begum 2003), and cytotoxic effects against human cancer cell lines (SCL, SCI-6, NUGC-4) and 3T6 fibroblast line (Nazrul Islam et al., 2004). Beside the cytotoxic effect of essential oil and ethyl acetate extracts of black seeds against different cancer cell lines, the injection of essential oil into solid tumor in an *in vivo* mouse model significantly decreased the tumor size, constrained the frequency of liver metastasis and amended the survival chance of the mouse (Ait Mbarek et al., 2007). Randhawa and Alghamdi (2011) reported that the mechanism by which *N. sativa* and thymoquinone damage cancer cells include mechanisms associated to tumor cell death and inhibition of proliferation, and mechanisms of inhibiting tumor angiogenesis, invasion and metastasis.

The present study aims to induce and maintain callus formation from leaf explants of *N. sativa.* Compare the thymol production from seedling and callus cultures. Investigate the effect of seedling and callus extract on two different cancer cell lines, human breast adenocarcinoma (MCF-7) and human colon cancer (Caco-2).

MATERIALS AND METHODS

This study was conducted in Tissue Culture Research Laboratory, Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt during the years from 2017 to 2018. This study does not contain any experiments on human or animal subjects.

Plant material and surface sterilization

Seeds of black seed *(N. sativa)* were obtained from Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. Seeds were initially exposed to sanitary soapy (Septol) water solution for 10 min, then to Dettol solution (3%) for 5 min and rinsed for one hour under running tap water. Under the aseptic condition, seeds were immersed in 70% ethanol for one minute. Subsequently; the seeds were surface disinfected with 1 or 2% sodium hypochlorite prepared from commercial bleach with a few drops of Tween-20 for 5 and 10 minutes.

Callus initiation

For seed germination, the basal salts mixture of MS medium (Murashige and Skoog 1962) supplemented with 25 g/l sucrose and pH adjusted to 5.7±0.1 was solidified with 7 g/l agar (Sigma-Aldrich, St. Louis, MO, USA). All the culture treatments were incubated in a growth chamber at 25±1 °C 16/8 h light/dark photoperiod (illumination intensity of 20 µmol m^{-2} s^{-1} using 40 watts white cool fluorescent lamp).

Table 1. Plant growth regulators (PGRs) treatments for callus induction in *N. sativa*

Treatments	Plant growth regulators (mg L^{-1})*			
	TDZ	2,4-D	NAA	BAP
1-	2	-	-	-
2-	-	2	-	-
3-	-	-	2	-
4-	-	-	-	-2
5-	4	-	-	-
6-	-	4	-	-
7-	-	-	4	-
8-	-	-	-	4
9-	-	2	-	2
10-	-	4	-	2
11-	-	-	2	2
12-	-	-	4	2

* TDZ = Thidiazuron [1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea] (TDZ), BAP = 6-benzylaminopurine, NAA = α-naphthalene Acetic acid and 2,4-D = 2,4-Dichlorophenoxyacetic acid.

For callogenesis, leaf explants (0.5 cm^2) were excised from five weeks old seedlings of black seed. For initiation of callus formation, the explants were cultured on MS medium supplemented with different plant growth regulators (Table 1). Calli were induced under 20 µmol m^{-2} s^{-1} illumination condition. All cultures were examined after five weeks of incubation at 25±1 °C under 16/8 h light/dark photoperiod.

Callus treatments and growth

Callus initiated from leaf explants of *N. sativa* were divided into one gram fresh biomass portions and cultured onto MS medium supplemented with different concentrations of Thidiazuron [1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea] (TDZ) (1, 2 or 3 mg L^{-1}) or BAP (6-benzylaminopurine) (1, 2 or 3 mg L^{-1}) combined with 2 mg L^{-1}NAA (α-Naphthalene Acetic Acid). Incubation was carried out under three different illumination condition (dark incubation, low intensity of 10 µmol m^{-2} s^{-1} and high intensity of 20 µmol m^{-2} s^{-1}) provided by cool fluorescent light. Incubation temperature was adjusted to 25±1ºC. Callus was subcultured every five weeks for three successive cycles. Ten replicates represented each treatment. The growth of calli was measured by the amount of both fresh and dry biomass of calli produced per single explant. Calli were harvested on a sterile dry filter paper, weighed to determine fresh weight and then placed in hot air oven at 70 $^{\circ}C$ until constant weight was obtained to determine the dry biomass per callus.

Determination of thymol content

Thymol content was determined by HPLC according to (Pandey et al. 2018). For extraction, one gram of two samples of dry powder of callus cultured on MS media supplemented with 1 mg L^{-1} TDZ, 2 mg L^{-1} TDZ and one sample of germinated seeds were separately homogenized into 10 mL of 80% methanol and shacked for 24h at room temperature. Methanolic extract of each sample was filtered by Whatman filter paper no. 1. The filtrate (extract) was evaporated under reduced pressure and the residue was stored at -20 °C for further analysis. HPLC analysis was performed using a C18 column. The mobile phase consisted of water: acetonitrile (50:50 v/v) at a flow rate 1.5 mL/min. The injection volume was 10 µl from each sample solution. The column temperature was maintained at 25 °C. The multi-wavelength detector was monitored at 280 nm. The quantity of thymol compound was calculated by peak areas compared to a peck area of an authentic sample of thymol and results were expressed as µg g^{-1} callus dry weight. The thymol authentic sample was purchased from Sigma–Aldrich.

Cytotoxicity Human cell culture

Two different human cancer cell lines, MCF-7 and Caco-2 were obtained from ATCC (Rockville, MD, USA). The cells were cultured in a humid environment at 37 °C and 5% CO_2 in minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen). At 85-90% confluence, cells were harvested using 0.25% trypsin/EDTA solution and sub-cultured onto 96-well plates according to the experimental requirements (Mosmann 1983).

The cells viability was determined by MTT assay according to Mosmann (1983) with some modification to screen the cytotoxic activity of plant extracts. This assay was carried out at Science Way for Scientific Research and Consultations (Cairo, El-Mokatum, Egypt). Briefly, the 96 well tissue culture plate was inoculated with 1 X 10^5 cells/ml (100 µl/well) and incubated at 37 °C for 24 h to develop a complete monolayer sheet. Growth medium was poured from 96 well micro plates after confluent layer of cells were formed, and cell monolayer was washed twice with wash media. Two-fold dilutions of the tested sample in Roswell Park Memorial Institute (RPMI) medium (Moore and Woods 1977) with 2% serum (maintenance medium) was prepared. Dilution of 0.1 ml of each extracted sample was tested in different wells leaving 3 wells as control, which received only maintenance medium. Plates were incubated at 37 °C and the cells were examined for any physical signs of toxicity, e.g., partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. MTT solution was prepared (5 mg/ml in PBS) (Bio Basic Canada Inc, Markham, ON, Canada). 20 µl MTT solutions were added to each well and shaked at 150 rpm for 5 minutes, to thoroughly mix the MTT into the media. Afterward, the plates were incubated at 37 °C with 5% CO_2 for 1-5 hours to allow the MTT to be metabolized. After incubation was completed, the media was dumped off from the plates and allowed to dry on paper towels to remove residue if necessary. Resuspend formazan (MTT metabolic product) in 200 µl Dimethyl Sulfoxide (DMSO) and placed on a shaking table at 150 rpm

for 5 minutes to thoroughly mix the formazan into the solvent. Read optical density at 560 nm and subtract background at 620 nm. Optical density should be directly correlated with cell quantity.

Statistical analysis

All the experiments were set up in a completely randomized design and performed in at least triplicate. The data were subjected to one-way analysis of variance (ANOVA). Significant differences between mean values were detected using Fisher LSD. All the statistical analysis was done using the SPSS statistical package for Windows (Version 20; SPSS Inc., Chicago, USA) statistical software package.

RESULTS AND DISCUSSION

Surface sterilization of seeds

Surface sterilization of *N. sativa* seeds was carried out successfully as illustrated in Table (2). It is clear that the increase in the concentration of commercial bleach used or the duration of treatment was accompanied by an increase in the surface sterilization efficacy, which result in increased in the survival percentage of seedling (Fig. 1A). Using 2% commercial bleach for 10 minutes is probably an advisable choice where the percentage of healthy sterile plantlets reached up to 71.43%, which may be considered acceptable. These results agreed with earlier studies that successfully used commercial bleach for surface sterilization for different plant system such as *Psidium guajava* (Awad 1993), *N. sativa* (Al-Ani 2008; ElNour et al., 2015), *Dillenia indica* (Abd El-Kadder et al., 2014), and *Rosmarinus officinalis* (Hussein et al., 2017).

Callus initiation

Callus initiation and vigor of growth varied according to the type and concentration of plant growth regulators used (Table 3). Using TDZ alone at 2 mg L^{-1} resulted in the formation of callus with a good vigor of growth (+++) (Fig. 1B) while the higher concentration (4 mg L^{-1}) resulted in a poor callus formation (+) (Table 3). BAP, 2,4-D (2,4-Dichlorophenoxyacetic Acid) and NAA at either low (2 mg L^{-1}) or high (4 mg L^{-1}) concentrations could not result in inducing callus formation from *N. sativa* explants at all while combining BAP and NAA at 2 or 4 mg L^{-1} each could result in the formation of some callus morphogenesis (Table 3). The reason that 2,4-D could not induce callus formation in the present study and also other studies like (Al-Ani 2008) is probably due to the use of inappropriate concentration. Phua et al., (2016) reported that the auxin 2,4-D is an effective plant cell regulator in inducing cell elongation and enlargement commonly used for callus induction, however, when they use it to initiate callus from Sabah snake grass (*Clinacanthus nutans)* explants, they found that all concentrations of 2,4-D above 1 mg L^{-1} failed to induce viable callus with significant amount of fresh weight. Recent study by Kachhap et al., (2018) stated that maximum callusing of *Ocimum sanctum* was observed in MS medium supplemented with 0.1 mg L^{-1} 2, 4-D and decreased at higher concentrations (0.2, 0.4, 0.6, 0.8, 1 mg L^{-1}). Initiation of callus was totally arrested when explants were grown in MS medium supplemented with 1.2 or 1.4 mg L^{-1} of 2, 4-D. However, Abd El-Naby (2006) was successfully able to use high 2,4-D concentrations 4 mg L^{-1} plus BAP at 2 mg L^{-1} to induce callus from *Balanitis aegyptiaca* explants. Ibrahim et al. (2015) found that using 2,4-D at 1 mg L^{-1}combined with kinetin at 1.5 mg L^{-1} was an ideal formulation in inducing callus from stem, and leaf explants (100%) and root explants (90%) of *N. sativa*.

Table 2. Effect of concentration and exposure time of sodium hypochlorite on surface sterilization of *N. sativa* seeds cultured *in vitro*.

Concentration of Sodium hypochlorite	Exposure time (min)	Contaminated plantlets (%)	Sterile healthy plantlets (%)
1%	5.0	100.00	0.00
	10.0	50.00	50.00
2%	5.0	83.33	16.67
	10.0	28.57	71.43

Table 3: Effect of plant growth regulators on leaf disc explants of *N. sativa* to initiate callus growth.

Treatments*	Vigor of callus growth
Hormone free MS	-
TDZ, 2 mg L^{-1}	**+++**
TDZ, 4 mg L^{-1}	**+**
BAP, 2,4-D or NAA at 2 mg L^{-1} separately	-
BAP, 2,4-D or NAA at 4 mg L^{-1} separately.	-
2,4-D, 2 mg L^{-1} + BAP, 2 mg L^{-1}	-
2,4-D, 4 mg L^{-1} + BAP, 2 mg L^{-1}	-
NAA, 2 mg L^{-1} + BAP, 2 mg L^{-1}	**++**
NAA, 4 mg L^{-1} + BAP, 2 mg L^{-1}	**+**

* TDZ = Thidiazuron [1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea] (TDZ), BAP = 6-benzylaminopurine, NAA = α-naphthalene Acetic acid and 2,4-D = 2,4-Dichlorophenoxyacetic acid

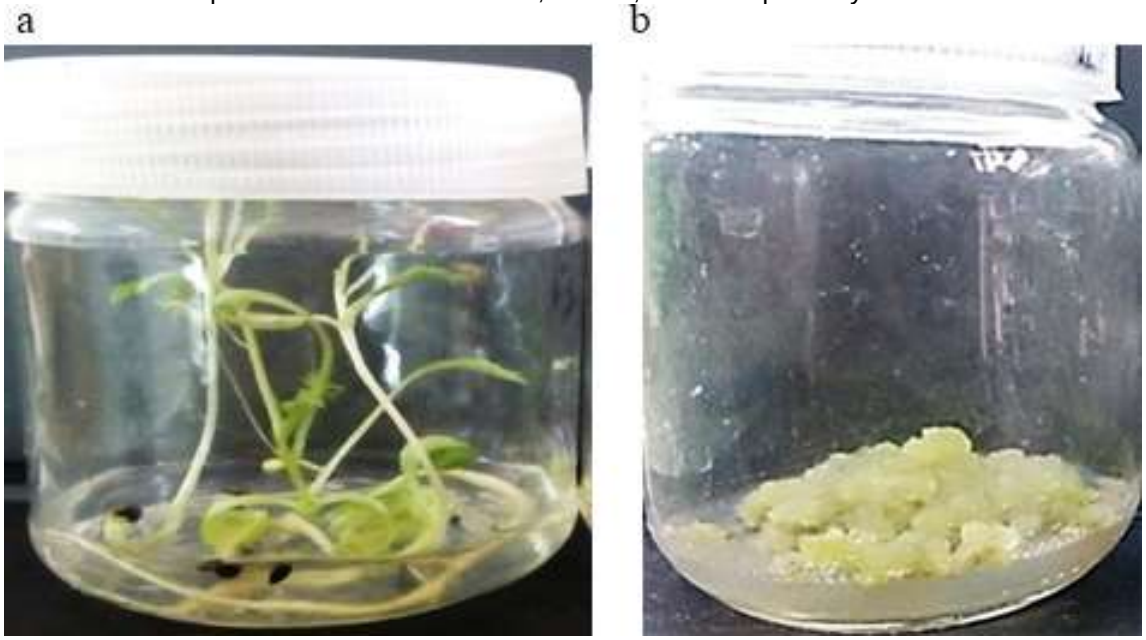

Figure 1. a) Aseptic germination of *N. sativa* on hormone free MS basal salts after five weeks of culture and b) vigorous callus growth on MS media supplemented with 2 mg L^{-1} TDZ.

Callus growth

Results presented in Table (4) and Fig. (2) show that the fresh weight per callus ranged from 2.88±0.06 in calli treated with 1 mg L^{-1} BAP + 2 mg L^{-1} NAA at dark to 12.28±0.98 gram per callus with 2 mg L^{-1} TDZ at 20 µmol m^{-2} s^{-1} illumination intensity, which is an approximately four folds of the minimum fresh weight obtained. From the results illustrated in Table (4), it seems that TDZ alone was superior to BAP combined with NAA treatments in enhancing fresh weight accumulation and that light, in general, played a decisive role in calli growth. The presence of light was generally accompanied by an increase in the fresh weight of calli with concomitant greening of calli (Fig. 2), and the effect was much more pronounced with TDZ over BAP plus NAA treatments. Green calli had better weight than corresponding white calli. It is possible that green calli exhibit better ability to do photosynthesis and accumulate more biomass. Green calli may also have better potential to synthesize not only primary metabolites but also secondary metabolites because many of the intermediate compounds formed in primary metabolism can also trigger secondary metabolism. The common observation also may be that the slight decline in fresh weight over subculturing. It means under the same conditions, the fresh weights in second subculture were less than that corresponding weights in the first subculture and the fresh weights in the third subcultures were lower than their similar weights in the second subculture.

In the first subculture on the same PGRs treatments, with increasing light intensity from zero, 10 to 20 µmol m^{-2} s^{-1}, the callus growth in term of fresh weight takes in increased direction. During the second subculture, the highest significant fresh weight at dark, 10 µmol m^{-2} s^{-1}, and 20 µmol m^{-2} s^{-1} illumination condition was (7.86, 8.21 and 9.78 g, respectively) when calli were grown on MS medium supplemented with 2 mg L^{-1} TDZ (Table 4). The lowest significant fresh weights at dark, 10 µmol m^{-2} s^{-1}, and 20 µmol m^{-2} s^{-1} illumination conditions (3.13, 4.19 and 4.35 g, respectively) were observed on MS medium amended with 1 mg L^{-1} BAP plus 2 mg L^{-1} NAA (Table 4). During third subculture under light intensity conditions (10 and 20 µmol m^{-2} s^{-1}) all treatments of plant growth regulators tested exhibited insignificant effects on calli fresh weights, while in dark incubation condition the highest significant fresh (5.95 g per callus) was obtained when callus culture was grown on MS medium supplemented with 2 mg L^{-1} TDZ (Table 4).

As regards to dry weight (Table 5), callus dry weight significantly affected by plant growth regulators under the three light intensity illumination conditions examined (Dark, 10 and 20 µmol m^{-2} s^{-1}). In general, the calli obtained using MS medium supplemented with 2 mg L^{-1} TDZ had the highest significant dry weight among all plant growth regulators tested. The highest significant dry weight in full dark, 10 µmol m^{-2} s^{-1} and 20 µmol m^{-2} s^{-1} during first subculture (0.27, 0.32 and 0.52 g, respectively), second subculture (0.24, 0.26 and 0.45 g, respectively) and third subculture (0.21, 0.21and 0.27 g, respectively) were obtained when callus culture was grown on MS medium supplemented with 2 mg L^{-1} TDZ (Table 5). Study the effect of TDZ on the growth of callus culture of *N. sativa* was rare. Al-Ani (2008) obtained the best callus production from leaf explants of *N. sativa* by using 1 mg L^{-1} 2,4-D combined with 1.5 mg L^{-1} Kinetin. Also, (Chaudhry et al., 2014) reported that epicotyl region of *N. sativa* cultured on MS media supplemented with 2 mg L^{-1} Kinetin plus1 mg L^{-1} NAA produced fast growing creamy and friable callus. TDZ is a substituted phenylurea compound which was developed for mechanized harvesting of cotton bolls and has now emerged as a highly effective bioregulator of morphogenesis in the tissue culture of many plant species (Murthy et al. 1998). Application of TDZ induces a diverse array of cultural response. Furthermore, Murthy et al. (1998) stated that TDZ exhibits the unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants. This may explain what was observed in the present study that TDZ alone exceeded the combined effect of BAP and NAA on callus fresh weight, dry weight, and pigment formation.

Thymol content in callus and germinated seedling

Thymol is one of the major components present in *N. sativa* seed oil about 10.2% (Kazemi 2014). HPLC histograms for thymol determination is presented in Fig. (3). The thymol content in callus subjected to plant growth regulators (Fig. 3C, D) compared with *in vitro* germinated seeds (Fig. 3B) which used as the mother plant. Data revealed that the highest thymol content (521.88 µg g^{-1} dry weight) was recorded in germinated seeds (Fig. 3B) followed by callus cultured on MS media supplemented with 2 mg L^{-1} TDZ (118.74 µg g^{-1} dry weight) (Fig. 3D) and the lowest thymol content (74.41 µg g^{-1} dry weight) was documented in callus cultured on MS media supplemented with 1 mg L^{-1} TDZ (Fig. 3C). Al-Ani (2008) reported that the higher thymol concentrations were extracted after 75 days for the callus which obtained from leaf explants of *N. sativa*. Also, Chaudhry et al. (2014) reported that thin layer chromatography (TLC) of *N. sativa* callus cultured on MS media supplemented with 2 mg L^{-1} Kinetin + 1 mg L^{-1} NAA and 2 mg L^{-1} BAP + 1 mg L^{-1} IAA had recorded presence of thymol with a characteristic pink spot of R_f value 0.76. In another study, Ibrahim et al. (2015) found out that silver nitrate and salicylic acid chemical abiotic elicitors proved to enhance the synthesis of secondary metabolites like fatty acids and essential oil in *N. sativa* callus culture. The results obtained in the present study may represent a shred of additional evidence that callus cultures are a promising alternative for the production of valuable phytopharmaceutics in the future.

Cytotoxicity of human cell culture

In this study, an initial screening of methanol extracts of calli and germinated seeds of *N. sativa* was carried out against the human breast adenocarcinoma cell line (MCF-7) and human colon cancer cell line (Caco-2). The results of cell viability assay revealed that the methanol extract used from germinated seeds and callus extract has anticancer action on (MCF-7) and (Caco-2) cell lines (Fig. 4).

Table 4. Fresh weights of calli (in grams) in response to different growth regulators and illumination intensities applied for three successive subcultures.

Treatments* (mg L^{-1})			Callus fresh weight (g)								
			1st Subculture			2nd Subculture			3rd Subculture		
BAP	NAA	TDZ	Dark	10 µmol m^{-2} s^{-1}	20 µmol m^{-2} s^{-1}	Dark	10 µmol m^{-2} s^{-1}	20 µmol m^{-2} s^{-1}	Dark	10 µmol m^{-2} s^{-1}	20 µmol m^{-2} s^{-1}
1	2	-	2.88±0.06^{c}	7.45±1.36^{a}	5.81±1.27^{b}	3.13±0.32^{d}	4.19±0.57^{b}	4.35±0.49^{b}	3.01±0.62^{bc}	4.21±0.23^{a}	3.82±0.89^{a}
2	2	-	3.37±0.34^{bc}	8.38±1.14^{a}	6.47±1.21^{b}	3.41±0.12^{cd}	4.45±0.41^{b}	4.82±0.96^{b}	1.91±0.21^{c}	4.24±0.43^{a}	3.54±0.37^{a}
3	2	-	4.27±0.59^{bc}	7.49±0.43^{a}	6.40±0.20^{b}	3.81±0.07^{bc}	5.20±0.59^{b}	5.49±0.10^{b}	4.08±0.56^{ab}	4.14±0.58^{a}	4.29±0.51^{a}
-	-	1	5.33±0.59^{b}	9.80±0.86^{a}	10.49±0.49^{a}	4.84±0.51^{b}	5.90±0.33^{b}	8.40±0.22^{a}	3.33±0.28^{bc}	3.67±0.38^{a}	4.74±0.95^{a}
-	-	2	8.77±1.25^{a}	10.22±0.16^{a}	12.28±0.98^{a}	7.86±0.58^{a}	8.21±0.68^{a}	9.78±0.24^{a}	5.95±1.40^{a}	7.85±2.17^{a}	6.71±1.25^{a}
-	-	3	5.00±0.87^{bc}	8.56±1.32^{a}	6.91±0.54^{b}	3.58±0.52^{cd}	5.95±0.97^{b}	5.11±0.59^{b}	4.43±0.32^{ab}	3.39±0.47^{a}	4.32±0.62^{a}
P value			***	NS	***	***	***	***	**	NS	NS
F ratio			8.375	1.364	9.196	18.097	5.352	18.339	4.010	2.844	1.894

Each value is a mean of 3 determinations ± the standard error, columns with the same letters are not significantly different according to Fisher LSD. NS = non-significant, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$.

* TDZ = Thidiazuron [1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea] (TDZ), BAP = 6-benzylaminopurine, NAA = α-naphthalene Acetic acid and

Table 5. Fresh weights of calli (in grams) in response to different growth regulators and illumination intensities applied for three successive subcultures.

Treatments* (mg L^{-1})			Callus dry weight (g)								
			1st Subculture			2nd Subculture			3rd Subculture		
BAP	NAA	TDZ	Dark	10 µmol m^{-2} s^{-1}	20 µmol m^{-2} s^{-1}	Dark	10 µmol m^{-2} s^{-1}	20 µmol m^{-2} s^{-1}	Dark	10 µmol m^{-2} s^{-1}	20 µmol m^{-2} s^{-1}
1	2	-	0.05±0.02^{d}	0.19±0.01^{bcd}	0.24±0.003^{c}	0.11±0.01^{c}	0.16±0.03^{a}	0.19±0.01^{c}	0.093±0.01^{b}	0.15±0.04^{a}	0.17±0.03^{a}
2	2	-	0.12±0.01^{c}	0.16±0.01^{cd}	0.24±0.03^{c}	0.10±0.01^{c}	0.11±0.03^{a}	0.16±0.03^{c}	0.09±0.02^{b}	0.11±0.02^{a}	0.16±0.03^{a}
3	2	-	0.16±0.02^{bc}	0.12±0.03^{d}	0.21±0.04^{c}	0.11±0.01^{c}	0.15±0.03^{a}	0.22±0.01^{c}	0.09±0.02^{b}	0.11±0.03^{a}	0.21±0.03^{a}
-	-	1	0.19±0.01^{b}	0.21±0.02^{bc}	0.46±0.02^{a}	0.16±0.01^{b}	0.16±0.03^{a}	0.32±0.05^{b}	0.11±0.04^{b}	0.12±0.02^{a}	0.20±0.06^{a}
-	-	2	0.27±0.02^{a}	0.32±0.04^{a}	0.52±0.01^{a}	0.24±0.01^{a}	0.26±0.05^{a}	0.45±0.02^{a}	0.21±0.02^{a}	0.21±0.06^{a}	0.27±0.03^{a}
-	-	3	0.15±0.02^{bc}	0.25±0.02^{ab}	0.37±0.03^{b}	0.15±0.01^{b}	0.12±0.01^{a}	0.35±0.03^{b}	0.13±0.00^{b}	0.08±0.01^{a}	0.18±0.03^{a}
P value			***	**	***	***	NS	***	**	NS	NS
F ratio			19.008	7.652	30.528	24.074	2.560	14.308	3.900	1.552	1.212

Each value is a mean of 3 determinations ± the standard error, columns with the same letters are not significantly different according to Fisher LSD. NS=non-significant, * = significant at $P < 0.05$, ** = significant at $P < 0.01$, *** = significant at $P < 0.001$.

* TDZ = Thidiazuron [1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea] (TDZ), BAP = 6-benzylaminopurine, NAA = α-naphthalene Acetic acid.

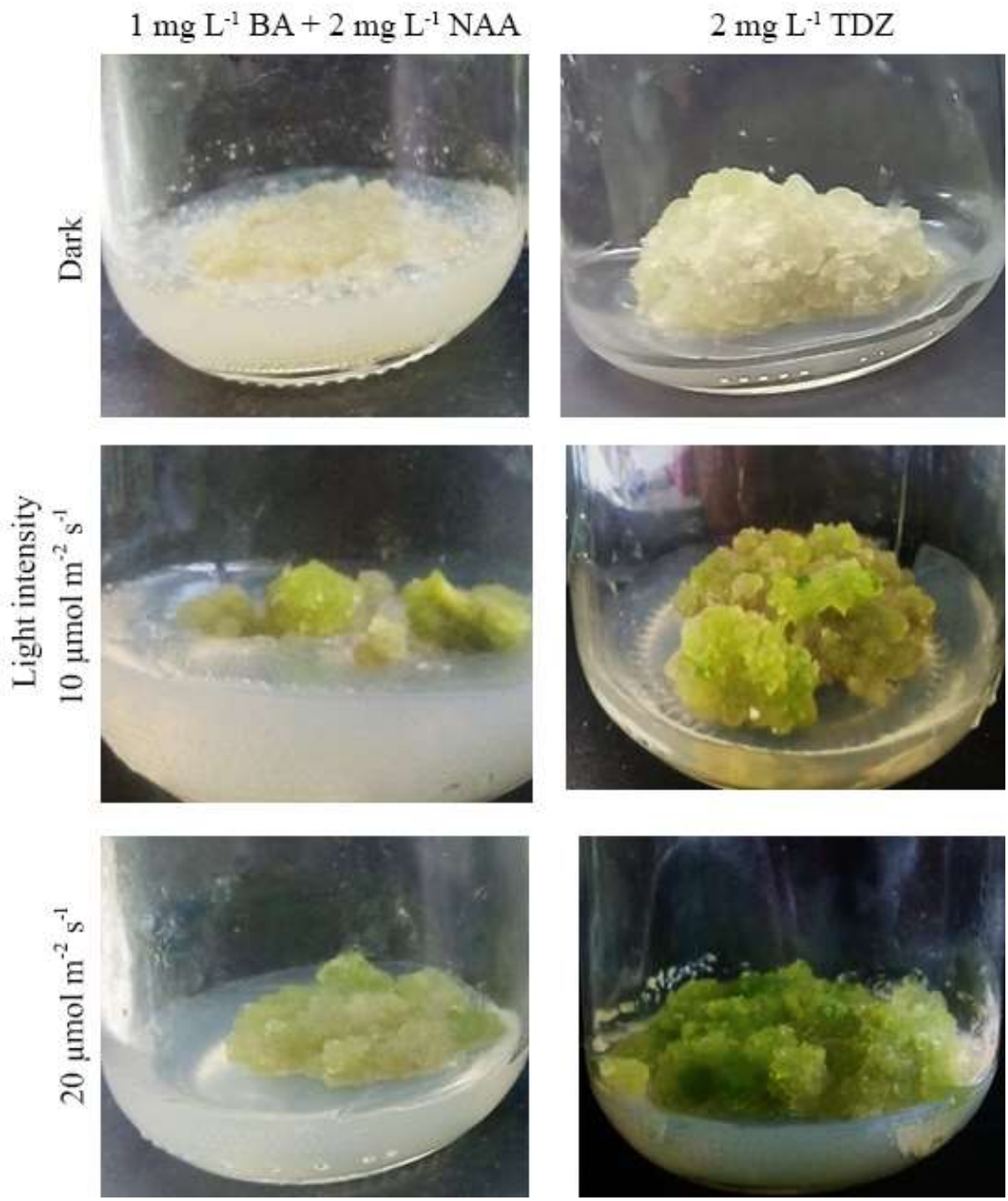

Figure 2. Calli of *Nigella sativa* produced after 5 weeks of growth on MS supplemented with different plant growth regulators under various illumination conditions.

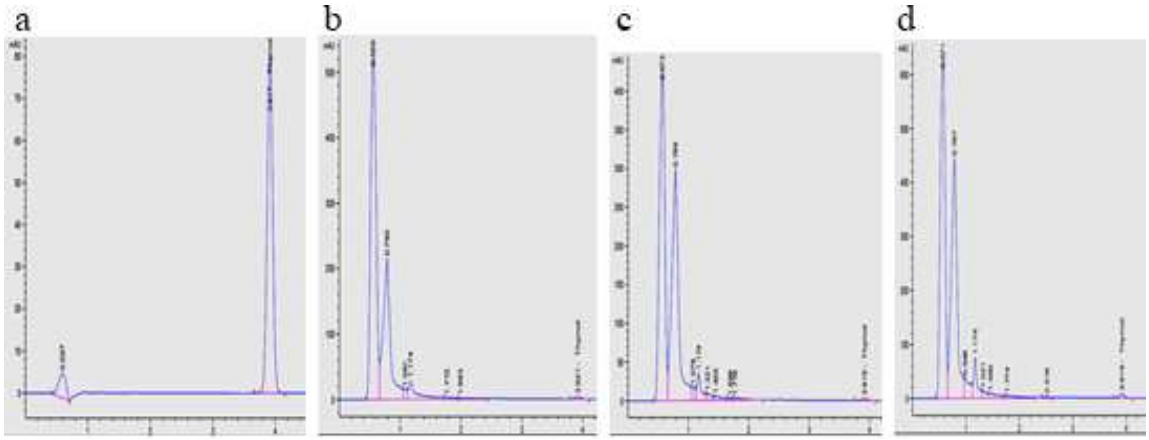

Figure 3. HPLC chromatograms representing thymol content in micrograms per gram dry plant powder. a) Authentic sample of thymol, b) Germinated seeds (521.89 µg g^{-1} dry weight), c) Callus cultured on MS media + 1 mg L^{-1} TDZ (74.41 µg g^{-1} dry weight) and d) Callus cultured on MS media + 2 mg L^{-1} TDZ (118.74 µg g^{-1} dry weight).

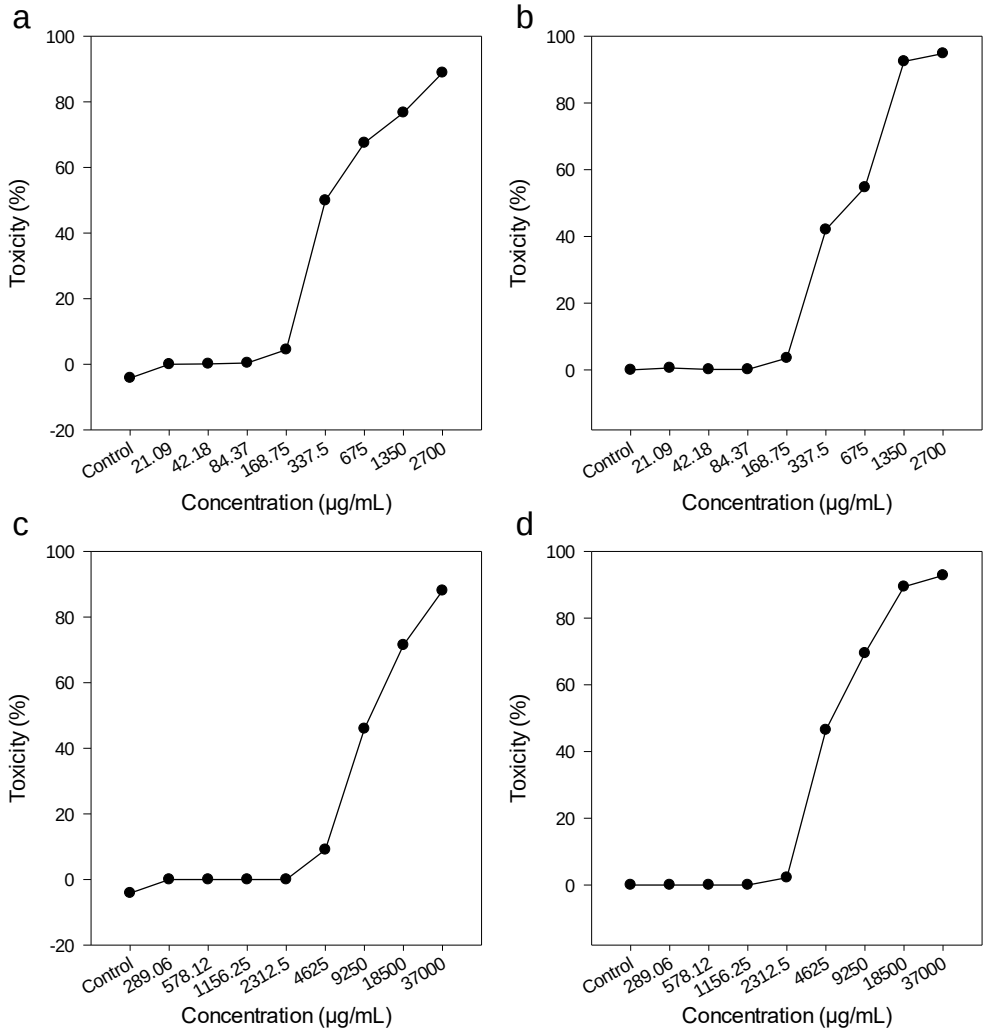

Figure 4. Effect of methanol extract of germinated seeds of *N. sativa* on cell proliferation of a) MCF-7 b) and Caco-2 at different concentrations. Effect of methanolic extract of *N. sativa* callus (maintained on MS media supplemented with 2 mg L^{-1} TDZ) on cell proliferation of c) MCF-7 and b) Caco-2 at different concentrations.

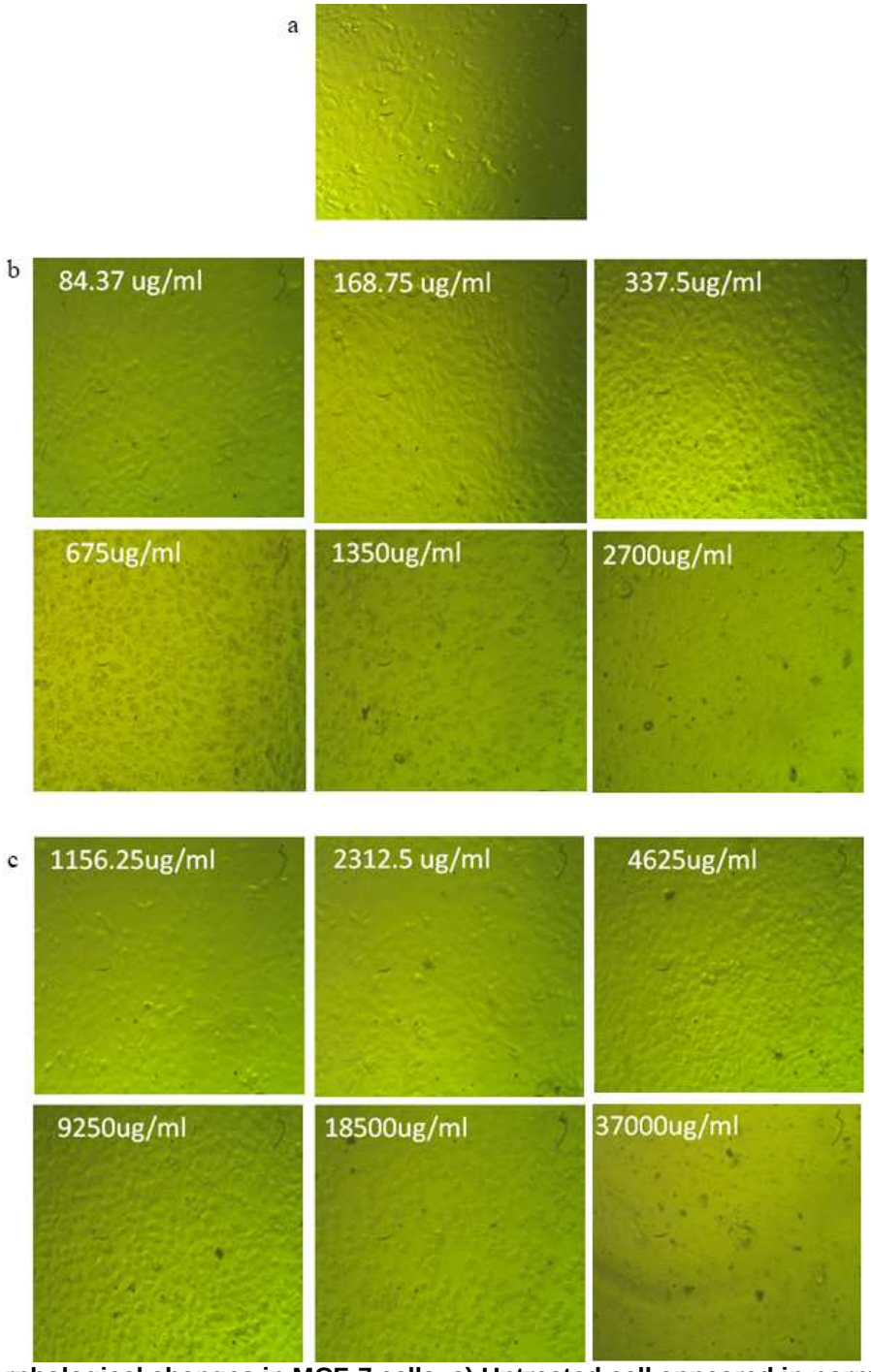

Figure 5. Morphological changes in MCF-7 cells, a) Untreated cell appeared in normal shape, b) MCF-7 cell exposed to different concentrations of methanolic extract of *N. sativa* germinated seeds, and c) MCF-7 cell exposed to different concentrations of methanolic extract of *N. sativa* (maintained on MS media supplemented with 2 mg L^{-1} TDZ).

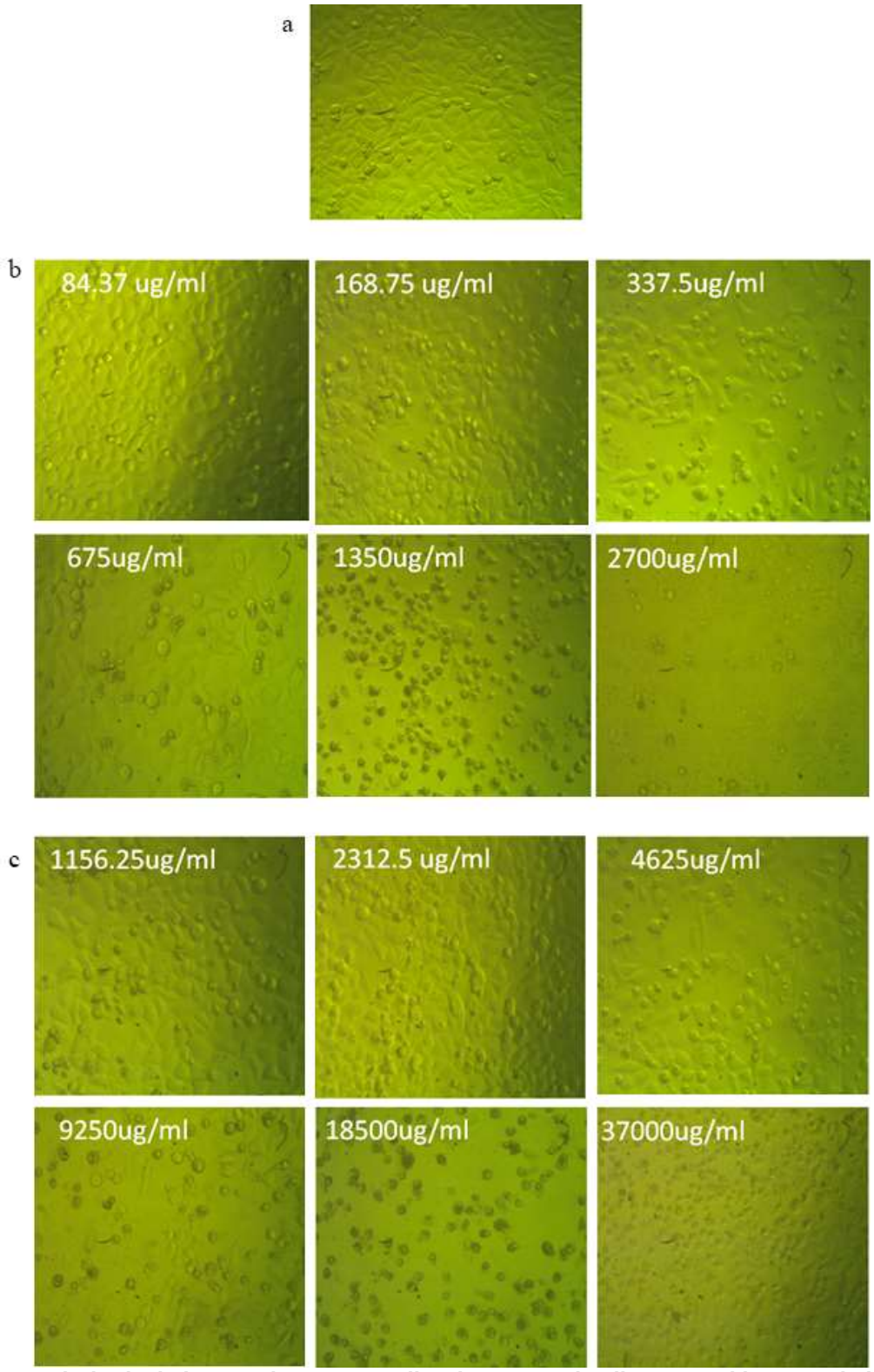

Figure 6. Morphological changes in Caco-2 cells, a) Untreated cell appeared in normal shape, b) Caco-2 cell exposed to different concentrations of methanolic extract of *N. sativa* germinated seeds, and c) Caco-2 cell exposed to different concentrations of methanolic extract of *N. sativa* (maintained on MS media supplemented with 2 mg L^{-1} TDZ)

The IC_{50} value was graphically obtained by plotting the percentage growth inhibition against the corresponding different concentrations of the test compound used. The obtained results clearly show that methanolic extract of germinated seeds exhibits promising cytotoxic activity on MCF-7 cell line with an IC_{50} value of 477 µg/ml (Fig. 4A) while the effect of the extract of germinated seed on Caco-2 cell line appears cytotoxic with an IC_{50} value of 688 µg mL^{-1} (Fig. 4B). Black seed preparations (TQ and TQ2) have been demonstrated to have significant antineoplastic activity against various tumor cells *in vitro* (Jafri et al. 2010). In breast cancer cells line MCF-7, thymol (0.05 – 1.25 µM) stimulated cytotoxicity by arresting the cell cycle in the G0/G1 phase (Jaafari et al., 2012). Thymol triggered cytotoxicity in MCF-7 breast cancer cell lines with an LC_{50} of 2.5 µg/mL (Melo et al. 2014). In another study, thymol present in the essential oil of *T. lanceolatus* (IC_{50} = 304.81 µg/mL) was shown to induce cytotoxicity and proliferation in MCF-7 cells (Khadir et al., 2016). The seed extracts from *N. sativa* have also been found active against DLD-1 colon carcinoma (Bourgou et al., 2012). In contrast, the methanolic extraction of callus culture showed cytotoxic activity on MCF-7 cell lines with an IC_{50} value of 1276 µg/ml (Fig. 4C), while the effect of the methanolic extract of callus culture on Caco-2 cell lines appears cytotoxic activity with IC_{50} value of 654 µg/ml (Fig. 4D). Gali-Muhtasib et al., (2004) studied the effect of Thymoquinone (TQ) on the HCT-116 (colon cancer cells line) and found that TQ is effective against colon cancer cells and trigger apoptosis in colon cancer cells in time and dose dependent manner thus inhibits the growth of cancer cells.

Morphological changes in untreated MCF-7 cells (Fig. 5A), which appeared in healthy shape, while alterations in the morphology of MCF-7 cells which exposed to germinated seeds extract (Fig. 5B) and callus extract (Fig. 5C) were found to be in a concentration-dependent manner. Cells exposed to 337.5 µg/mL and above concentrations of seed extraction reduced the normal morphology and cell adhesion capacity of MCF-7 cells (Fig. 5B). In the case of callus extract, the morphology of MCF-7 cells at 4625 µg/mL started to reduce the standard shape and cell adhesion capacity as compared to control. Similarly, Morphological changes in untreated Caco-2 cells (Fig. 6A), which appeared in reasonable shape, while alterations in the morphology of Caco-2 cells which exposed to germinated seeds extract (Fig. 6B) and callus extract (Fig. 6C) were found to be in a concentration dependent manner. Cells exposed to 337.5 µg/mL and above concentrations of germinated seeds extract extraction reduced the normal morphology and cell adhesion capacity of Caco-2 cells (Fig. 6B). In the case of callus extract, the morphology of Caco-2 cells at 4625 µg/mL started to reduce the standard shape and cell adhesion capacity as compared to control (Fig. 6C).

CONCLUSION

In the present study trials were done to initiate callus from mother explant source (germinated seedling) of *Nigella sativa* leaf explants on MS media supplemented with different plant growth regulators. determine of thymol content in germinated seedling and callus was achieved by HLPC and found that thymol content of germinated seeds was about four to seven folds higher than thymol content of callus extract. Methanol extract of germinated seedling and callus extract showed anticancer activity against human breast adenocarcinoma (MCF-7) and colon cancer (Caco-2) cell lines. The obtained results indicated that callus cultures exhibited the potential to synthesize thymol and exhibit some cytotoxic effects against the tested human cell lines. Moreover, the current study may open anew avenue for the promising future role that plant cell cultures may play on the production of valuable medicinal constituents *in vitro.*

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

MSA, callus experiments supervision, analyzed the results and wrote the callus culture part of the research. AKA performed the callus experiments and data collection. LII performed volatiles analysis and wrote the anticancer part of the research., EAH the research supervisor, data interpretation, and edited the manuscript.

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