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ARTICLE

Stabilizing Effects of Ethanolic Extract of Mastic Gum on Microtubule Polymers: an In Vitro Study

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ABSTRACT

Terpenoids are novel natural products isolated from mastic gum. Mastic gum was obtained from the Pistacia Lentiscus tree. Scientific investigations have documented medical and pharmacological properties of mastic gum such as memory enhancement, antifungal, and antibacterial activities. It was astonishing to study the possible interaction of mastic gum extract with microtubule proteins which are involved in memory and consciousness since the administration of mastic gum is evidenced in the improvement of brain functions. Since a number of studies have demonstrated the effect of microtubule dynamics on mammals' memory, in this study, we investigated the effect of Oxygenated Sesquiterpenes (OST) on microtubule polymerization in vitro. OST was purified from the ethanolic extract of mastic gum. The results revealed that OST induces microtubule polymerization; however, microtubule depolymerization was not affected and fluorometric assays showed conformational changes of tubulin in the presence of OST. We interestingly found that colchicine was unable to inhibit MT assembly in the presence of OST and OST was solely more efficient than the combination of OST with paclitaxel for elevating microtubule polymerization rate. We hope that OST could be a promising agent for memory enhancement and the treatment of neurodegenerative diseases as a novel tubulin-binding compound.

1. Introduction

Pistacia lentiscus belongs to the Anacardiaceae family which is found in the Mediterranean regions [1]. Mastic gum is "well-known" for its terpenoid and essential oil compounds. The major components of mastic gum are consisted of flavonoids, triterpenoids, Oxygenated Sesquiterpenes (OST), phenolic compounds and some essential oils which show antifungal and antibacterial activities, and have been used for the treatment of hypertension, dyspepsia, abdominal discomfort and patients suffering from peptic ulcers [2-6]. This study of mastic gum compounds originated from oriental traditional medicine papers which revealed wide usage of mastic gum for decreasing anxiety and stress [7], memory enhancement [7,8], antioxidant behavior [9] and anti-inflammatory activity [10]. At the other end of the spectrum, it has been shown that microtubule (MT) proteins, which particularly play essential structural and functional roles in brain cells, are involved in the memory system [11]. Therefore interaction

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of OST with MT protein was the focus of this study.

MT proteins are dynamic polymers in all eukaryotic cells which play key roles in a variety of cellular functions. They are composed of tubulin heterodimers including α-tubulin and β-tubulin which are able to be assembled from head to tail through binging to guanosine triphosphate (GTP) [12,13]. MTs are essential proteins performing crucial roles in various cell functions such as mitosis, cell motility and cellular transportation [14]. Axonal MTs are the major internal structures defining the external shape, polarity of the neuron and substance transportation including neurotransmitters [15]. MT stability is promoted by MT-associated proteins (MAPs) which bind to MT polymers and regulate the polymerization [13]. MT ends switch between growing and catastrophic state in vitro as well as in situ; this kind of behavior has been termed as dynamic instability [16]. Agents and drugs interfering with MT polymerization are able to have an impact on MT dynamics and functions [17].

Recent investigations indicated the crucial role of MT protein in the enhancement of memory. In 1993, Qian et al. have demonstrated abnormal MT polymerization of old rats' brains in comparison with the juvenile rats [18]. Other Researches in 2002 showed that the infusion of colchicines into the rat bilateral rostrocaudal location has caused impaired memory function, which was due to the dysfunction of MTs [19]. Subsequent analyses have also confirmed the above-mentioned behavior of MT proteins [11,20]. Since MT dysfunction and instability are prominent in neurodegenerative diseases, e.g. Alzheimer's, MT-stabilizing agents could compensate for the loss of MT organization and function [21]. The effect of crocin on MT polymerization and structure in comparison with paclitaxel was previously reported in our lab indicating the ability of crocin for enhancing MT polymerization and nucleation rate as well as disruption of MT dynamics through acting as a stabilizing agent [22].

In this study, we have been searching for an effective molecular ligand interacting with MT proteins, which could stabilize MT polymers without any toxic effects. Hence, the impact of the ethanolic extract of mastic gum on MT dynamics was examined. At least four different compounds were accordingly isolated from ethanolic extract of mastic gum which revealed the adequate potency to induce MT polymerization in a concentration-dependent manner. Optimization of a series of extracted compounds resulted in obtaining OST, which was selected for further investigation because it displayed a high potential to stimulate MT polymerization. Moreover, we investigated the effects of OST combination with colchicine and paclitaxel on MT dynamics.

2. Materials and methods

2.1 Reagents

Paclitaxel and Colchicine (Aldrich Chemical Co.) were dissolved in DMSO before any usages. Phosphocellulose P11 was obtained from Whatman (Florham Park, USA). All other chemicals were purchased from Sigma Chemical Co. All solutions were prepared with double distilled water and were kept at 4 ºC for further application.

2.2 Preparation and Identification of OST by HPLC and GC-Mass

Pistacia lentiscus was granted by Professor. S. M. Ghaffari, University of Tehran. Its fruits were collected and dried in darkness. The essential oils of mastic gum were obtained by the protocol described by Abidi et al. [23]. Four different compounds were isolated from the ethanolic extract of mastic gum by preparative HPLC. The most effective one was Oxygenated Sesquiterpene (OST) which has been mentioned in the following experiments. A C18 reverse-phase column (150 × 3.9 mm inside diameter) was used with a 4 μm Nova-Pack C18 cartridge (Water, Milford, MA). The mobile phase consisted of HPLC was acetonitrile and was run isocratically at a flow rate of 1 ml/min. The elution was monitored at 308 nm. According to terpenoid elution time, the peaks were collected and OST was identified as $C_{15}H_{24}O_2$ with a molecular weight of 236 Dalton assessed by GC-mass spectrometry described by Ammari et al. [8]. The data was not displayed.

2.3 Tubulin Purification

Tubulin was purified from the sheep's brain through two cycles of temperature-dependent polymerization-depolymerization based on the Miller and Wilson method [24] with slight modifications. The fresh brain was homogenized in PEM buffer (100 mM PIPES, 1 mM EGTA, 2 mM MgSO4, and 1 mM ATP, pH 6.9) and the resulting homogenate was centrifuged for 30 min at 30000 g. The tubulin in the supernatant was polymerized for 45 min with the presence of Mg^{2+}GTP and PMG buffer (100 mM PIPES, 2 mM MgSO4, 1 mM EGTA and 3.4 M glycerol, pH 6.9) was used as the polymerization buffer. MTs were pelleted by centrifugation (120,000g, 45 min), resuspended in PEM buffer 4°C for depolymerization process and then followed by one more cycle of polymerization-depolymerization for further purification. In order to eliminate residual MAPs and other remained impurities, tubulin was subjected to the anion exchange chromatography in a phosphocellulose column P11 using the method revealed by Weingarten et al [25]. Purified tubulin was confirmed by

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Coomassie Brilliant Blue staining of 10% SDS-PAGE and then stored at the liquid nitrogen. The protein concentration was measured using Bradford reagent (Bio-Rad, Hercules, USA) with bovine serum albumin as standard [26].

2.4 Microtubule Polymerization and Depolymerization Assay

The effect of OST on the MT polymerization-depolymerization process was determined by turbidometric assays. Therefore, tubulin was incubated in PEM buffer with a final concentration of 2 mg/ml. Different concentrations of the substrates including OST, Paclitaxel, and Colchicine were consequently added to each sample cuvette. The tubulin assembly process was monitored by measuring the absorbance at 350 nm every 20 seconds at 37ºC after the addition of 1 mM GTP using a spectrophotometer equipped with a temperature controller (Varian, Melbourne, Australia). For disassembly assays, MTs incubated either with OST or alone were kept at 4°C and the absorbance changes were monitored at 350 nm. The depolymerized MT was re-polymerized at 37°C and the process was measured again as described above.

2.5 Fluorescence Spectroscopy

8-anilino-1-naphthalenesulfonic acid (ANS) was used as a fluorophore to examine conformational changes of tubulin in the presence of OST. Tubulin (2 μM) was incubated in the presence of different concentrations of OST at 4 °C for 10 min. ANS (50 μM final concentration) was added to the tubulin-OST solution and incubated again for about 7 min. The emissions were monitored between 450-550 nm following excitation at 380 nm. All experiments were carried out at 4 ºC by Cary eclipse fluorescent spectrophotometer (Varian, Australia) and 2 μM tubulin was used in all measurements.

2.6 Transmission Electron Microscopy (TEM)

In-vitro polymerized MTs with and without OST (10 μM) were fixed on carbon-coated Farmavar-treated copper grids with 200 meshes. Each grid was then negatively stained with 10 μl uranyl acetate 1% solution. Excessive uranyl acetate was withdrawn with filter paper and sample grids were air-dried and observed using an HU-12A transmission electron microscope (Hitachi, Japan).

2.7 Cell Culture

Two human neuroblastoma cell lines, SK-N-MC and SK-N-BE (2), and fibroblast cell line, L929, were used as models for exploring the effects of OST on the brain cells. The SK-N-BE (2) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% v/v fetal bovine serum (FBS) (Gibco). SK-N-MC and fibroblast cell lines were grown in RPMI-1640 medium, supplemented with 10% FBS. The cells were maintained in a humidified incubator supplied with 5% CO2 and 95% air at 37°C. For measuring the viability of the cells in the presence of various concentrations of OST, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in a 96-well cell culture plate after incubation of the substrates for 24 hours. Therefore, absorbance at 570 nm was determined for each well using Powerwave XS2 microplate spectrophotometer (BioTek, USA). The inhibition concentration (IC50) was calculated by linear regression, performed on the linear zone of the dose-response curve for absorbance readings [27]. Data were calibrated to the appropriate calibration curve.

3. Results

3.1 Effect of OST on Tubulin Assembly and Disassembly in Vitro

Turbidometric assays were done in order to investigate the effect of OST on MT polymerization-depolymerization in vitro. Untreated MTs exhibited a temperature-dependent polymerization-depolymerization cycle as long as Mg^{2+}GTP was added. It is displayed that the addition of OST can promote MT polymerization in a concentration-dependent manner (2-10 μM) at 37 ºC (Figure 1). The re-polymerization assay demonstrated a similar result indicating the enhancement of the re-polymerization process in the presence of increasing concentrations of OST (Figure 2). Therefore, it is can be conluded that OST has no effect on formation of tubulin aggregates and normal assembly-disassembly cycle of tubulin proteins in the presence of highet concentration of OST was observed (Figure 2). Moreover, TEM was applied to visualize the MTs structure treated with OST. Electron micrographs showed that MTs were formed and organized longer in the presence of OST with inflated intensity in comparison with control MT polymers (Figure 3).

We additionally evaluated the combined effect of Paclitaxel and OST on MT dynamics. MT was polymerized in the presence of 0.1 mM Paclitaxel and 10 μM OST to compare the OST effects with Paclitaxel on tubulin assembly. Increased level of MT polymerization in the presence of OST was observed compared to Paclitaxel and a combination of OST and Paclitaxel (Figure 4). However, OST was not able to change the inhibitory effect of Paclitaxel on microtubule depolymerization.

To figure out the possible correlation between OST and

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colchicine sites on MT proteins, polymerization was conducted in the presence of 0.1 mM colchicine and 10 μM OST. The result has interestingly exceeded expectations. Turbidity of tubulin-OST complex rocketed about three-fold more than treatment with the combination of OST and colchicine (Figure 5).

3.2 Effect of OST on MT Dynamics at Steady State

The results revealed that the addition of OST on assembled microtubule solution at the equilibrium phase caused an increase in turbidity and the polymerization process initiated again to reach a new equilibrium state (Figure 6). It was comparable to the result obtained from the polymerization assay (Figure 1).

3.3 Effect of OST on the Tubulin Conformation

To investigate the tubulin structural changes in the presence of OST, ANS fluorescence studies have been done. Tubulin-ANS complex manifests strong fluorescence ability that is extremely and environmentally sensitive. It is used to probe the conformational state of the tubulin and to determine the nature of the interactions [28]. The results revealed that the ANS fluorescence emission of tubulin was increased by an increment of OST concentrations due to the changes in the hydrophobic environment around the tubulin protein (Figure 7).

3.4 The Cell Culture Results

After 24 h incubation of OST with three cell lines including SK-N-MC, SK-N-BE (2) and L929, the viability of them was determined above 80%. The morphological changes have not also occurred (the data was not shown) and the results have displayed no significant toxicity at the concentration ranged from 0.2 to 3.2 μM (Figure 8).

4. Discussion

MT proteins are dynamic polymers encompassing tubulin subunits whose functions and stability are supposedly correlated to brain maintenance especially memory function [29]. The MT dynamicity is essential for neuronal proper activity and protection from neurodegeneration [30]. It is sensitive to various chemical agents targeting tubulin or MT polymers. The primary action of these agents is to bind to specific sites on the tubulin in order to alter the polymerization process. Hence, finding a new stabilizing agent with low neurotoxicity and harmful effects might open a new horizon for the treatment of neurodegenerative diseases [21]. Hence, the effects of ethanolic extract of mastic gum, which some evidence confirms its neuroprotective properties [31], on the tubulin structure and function were evaluated in vitro.

The obtained outcomes indicated that OST could increase the rate of MT polymerization in a dose-dependent manner and could change the MT steady-state equilibrium. The assembly-disassembly process in the presence of OST revealed that no aggregate formation was induced even at high concentrations. TEM images could confirm the results obtained from the turbidimetric assay, which illustrated the abundance of MT polymers with no aggregates formation in the presence of OST compared to the control. MT polymer is composed of an unstable tubulin-GDP core and a stable tubulin-GTP cap at its two distinct ends. The gain and loss of GTP cap originate a phenomenon called dynamic instability which is crucial in various brain cells functions particularly in cognition and memory [32,33]. OST may stabilize MTs by conformational changes inducing tubulin to cause more stable tubulin-GTP caps. However, the results suggested that OST has not negatively affected MT dynamic instability and the depolymerization process. It is also possible that OST might reduce the rate of GTP hydrolysis or Pi release, preventing the tubulin-GTP cap loss in order to stabilize MT polymers.

Since Paclitaxel is an effective antimitotic agent binding to tubulin to suppress the MT catastrophic phase and to enhance nucleation and elongation phases of MT polymerization [34], the effect of Paclitaxel and OST along with their combinational effect on the tubulin assembly were compared. OST could enhance the MT polymerization process similar to paclitaxel; however, the evidence showed that OST has not disrupted MT dynamic instability and increased elongation rate significantly in contrast to Paclitaxel. It is indicated that the effect of paclitaxel on MT dynamic could not be inhibited in the presence of OST; however, the active sites for OST and paclitaxel might be the same or vicinal. Turbidimetry assay revealed that tubulin assembly increased in the presence of OST alone in comparison with the combination of paclitaxel and the same concentration of OST. Therefore, it seems that OST and paclitaxel may compete for binding to tubulin. On a whole, it was shown that OST indicated more efficacy rather than the combination of OST with Paclitaxel for enhancement of MT polymerization rate.

Colchicine is a natural toxic product and an effective inhibitor of tubulin assembly in vitro. Colchicine binds to tubulin to form a tight complex and induces tubulin conformational change and the tubulin-colchicine complex inhibits MT growth [35]. Controversial to our expectation, we observed that colchicine, which usually has a devastating impact on the MT polymer, could improve the stability of MTs when it was applied in the presence of OST. It seems that OST caused conformational changes in MT

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structure that may interfere in the colchicine binding site and inhibit the action of colchicine; however, it is clear-cut that colchicine has attenuated the stabilizing effect of OST on tubulin assembly.

The fluorescence intensity emission of tubulin was identified in order to investigate tubulin conformational changes in the presence of increasing concentrations of OST. ANS is a fluorescence probe utilized to study the surface hydrophobicity of the protein after interacting with a ligand. Therefore, the protein-ANS complex provides beneficial data about the possible alterations of the protein conformation [36]. The obtained outcome demonstrated that the complex of OST-tubulin affected the tubulin conformation due to the increment of ANS fluorescence emission of tubulin upon the addition of OST. In other words, OST could promote the exposure of tubulin hydrophobic regions, which have buried in the interior structure of the native tubulin, on the protein surface. The conformational changes of tubulin induced by OST binding could be an important factor for the enhancement of tubulin assembly.

In conclusion, although OST could stabilize MT polymers, it had no suppressive effects on their natural dynamic instability. We conclude that OST is a unique potent compound that could interact with tubulin in order to induce MT polymerization. Further studies are required to define OST efficacy for the treatment of neurodegenerative diseases.

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Appendixes

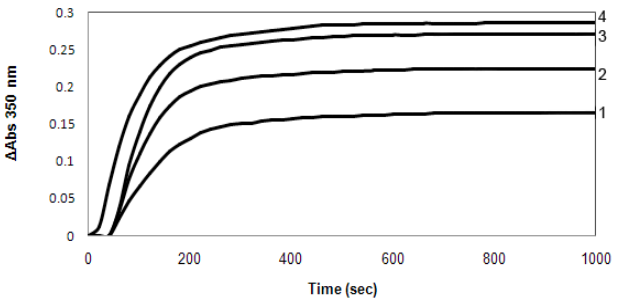


Figure 1. Effect of OST on MT Polymerization in vitro

Note: Tubulin (2 mg/ml) was incubated at 37 ℃ in the presence of 1 mM GTP, different concentrations of OST, and the same volume of solvent as a control. MT polymerization was monitored by measuring turbidity every 20 seconds at 350 nm. 1) 0 μM OST (Control); 2) 2 μM OST; 3) 5 μM OST; 4) 10 μM OST. The experiment was repeated four times.

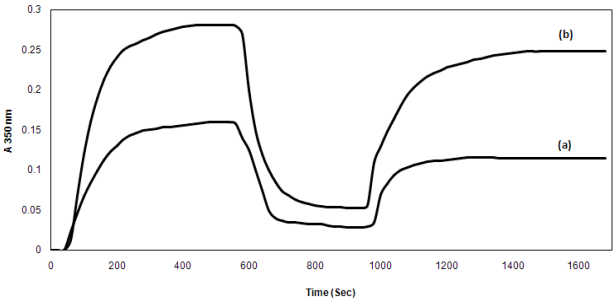
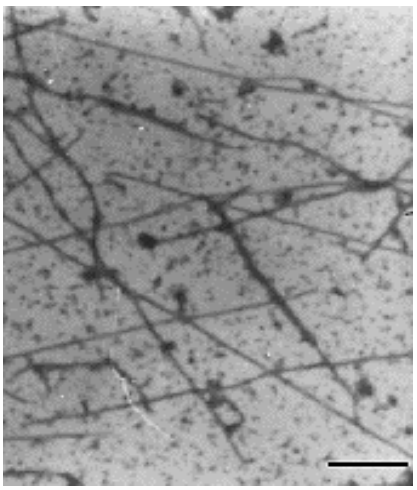
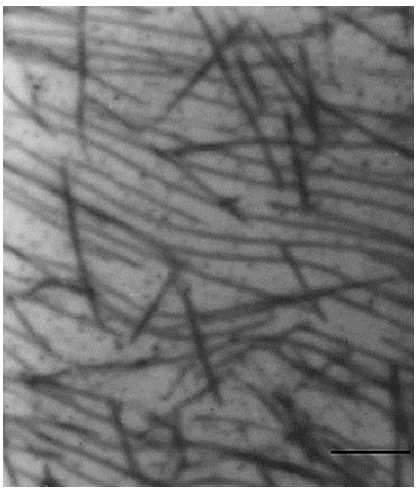


Figure 2. MT Repolymerization Assay

Note: Polymerized MT polymer (2 mg/ml) was disassembled by cooling down to 4 °C and then re-warming 37 ℃ to induce reassembly without any further addition of GTP or OST. Repolymerization was monitored by measuring the absorbance value every 20 seconds at 350 nm. a) 0 μM OST (Control); b) 10 μM OST.



(a)



(b)

Figure 3. Electron Microscopy Micrographs of MT Proteins

Note: MT proteins incubated with and without OST (10 μM) were negatively stained with 1% uranil acetate. a) MT polymers without OST; b) OST-treated MT polymers after 25 min of polymerization initiation. (Scale bars = 0.5 micrometer).

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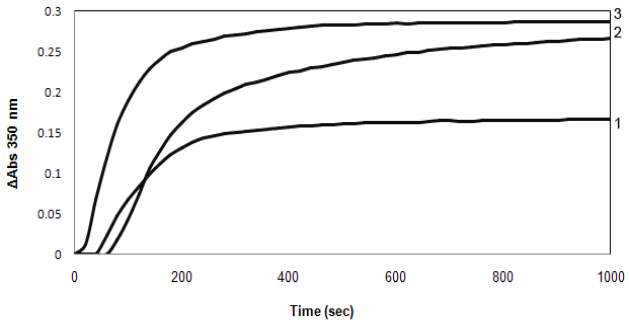

Figure 4. Tubulin Assembly Induced by OST and Paclitaxel

Note: Tubilin proteins (2 mg/ml) were incubated with OST, mixture of OST and Paclitaxel and equal volume of solvent as a control. Turbidity changes were observed every 20 seconds at 350 nm. 1) 0 μM OST and Paclitaxel (Control); 2) 10 μM OST and 0.1 mM Paclitaxel; 3) 10 μM OST.

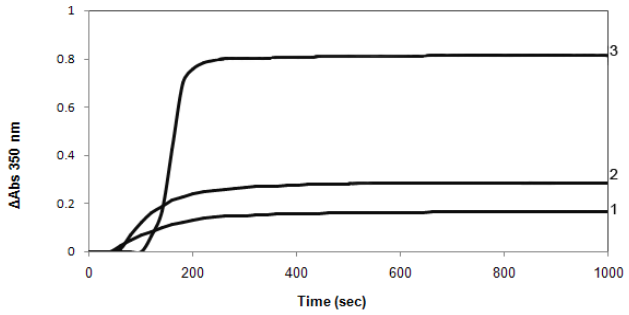

Figure 5. Tubulin Assembly Induced by OST and Colchicines

Note: Tubulin proteins (2 mg/ml) were incubated with OST, mixture of OST and Colchicine, and equal volume of solvent as a control. Turbidity changes were monitored every 20 second at 350 nm. 1) 0 μM OST and Colchicine (Control); 2) 10 μM OST and 0.1 mM Colchicine; 3) 10 μM OST.

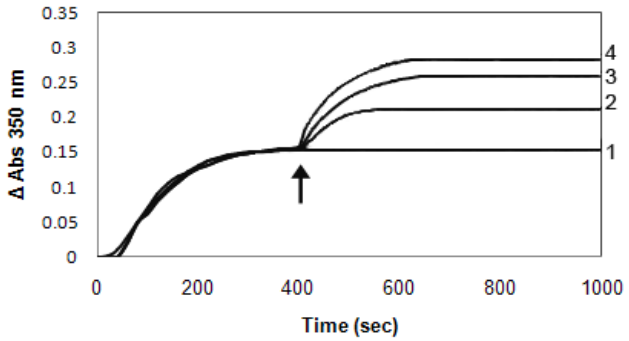

Figure 6. Effect of OST on MT Polymers at the Steady State Phase

Note: Tubulin proteins (2 mg/ml) were polymerized in the absence of OST. After 400 seconds from assembly initiation, various ranges of OST were added to the assembled tubulin in the steady state phase (displayed by the arrow). Polymerization process was recorded by turbidity changes at 350 nm. 1) 0 μM OST (Control); 2) 2 μM OST; 3) 5 μM OST; 4) 10 μM OST.

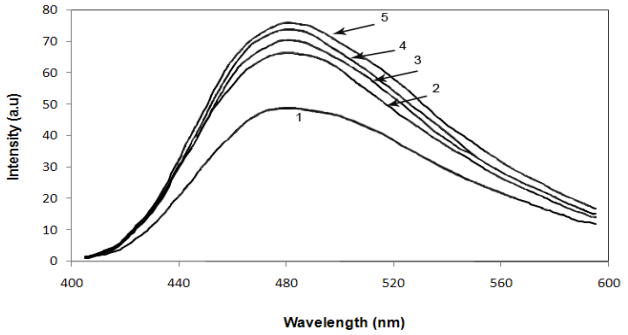

Figure 7. ANS Fluorescence Emission of Tubulin

Note: Tubulin proteins (2 μM) were incubated with various concentrations of OST (0, 2, 5, 7, 10 μM) for 10 min at 4 ℃. ANS (50 µM final concentration) was added and after 10 min incubation, fluorescence emissions were recorded from 400 to 600 nm following excitation at 380 nm. 1) 0 μM OST; 2) 2 μM OST; 3) 5 μM OST; 4) 7 μM OST; 5) 10 μM OST.

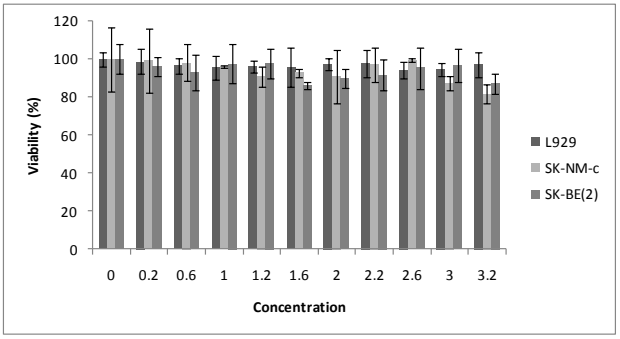

Figure 8. Viability Results of the Cell Culture Assesments

Note: SK-N-MC, SK-N-BE (2) and L929 Cells were treated with different concentrations OST ranged from 0.2 to 3.2 µM for 24 h and their viability was evaluated using MTT colorimetric assay.

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