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Cytotoxicity of *Usnea longissima* Ach. extracts and its secondary metabolite, usnic acid on different cells

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Usnea longissima Ach. ekstraktları ve onun sekonder metaboliti usnik asidin farklı hücreler üzerindeki sitotoksisitesi

Abstract: The biological activities of lichens, known as organisms based on a symbiotic relationship, are attracting more and more attention in traditional medicine and modern drug research. Lichens can possess various pharmacological effects such as antimicrobial, antioxidant, antitumor, anti-inflammatory, and many others due to the bioactive compounds they contain. In the present study, *Usnea longissima* Ach. and its secondary metabolite, usnic acid on human gastric adenocarcinoma cells (AGS), human colorectal adenocarcinoma cells (Caco-2), and mouse fibroblasts (NIH/3T3) were investigated. In this context, methanol and water extracts from *U. longissima* were obtained by Soxhlet extractor. The characterization of usnic acid was carried out by fourier transform infrared spectroscopy (FTIR). The cytotoxic activities of the extracts and the metabolite on cells were determined by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) analysis. Considering the median inhibitory concentration (IC_{50}) values, the application with the greater effect on AGS and NIH/3T3 cells was the methanol extract (373.17 µg/ml and 318.81 µg/ml, respectively). Considering the Caco-2 cells, it was determined that the water extract had the lowest IC_{50} value (230.05 µg/ml). The high cytotoxic activity of usnic acid on cancer cells (AGS; IC_{50}: 395.03 µg/ml and Caco-2; IC_{50}: 462.35 µg/ml) compared to normal cell (NIH/3T3; IC_{50}: 472.41 µg/ml) was noted. As a result, it has been revealed that methanol and water extracts of *U. longissima*, especially usnic acid, are products that can be used within the scope of complementary therapy.

Key words: Anticancer, extract, lichen, secondary metabolite

Özet: Simbiyotik bir ilişkiye dayanan organizmalar olarak bilinen likenlerin biyolojik aktiviteleri, geleneksel tıp ve modern ilaç araştırmalarında giderek daha fazla ilgi çekmektedir. Likenler, içerdikleri biyoaktif bileşikler sayesinde antimikrobiyal, antioksidan, antitümör, antiinflamatuar ve diğer pek çok farmakolojik etkiye sahip olabilir. Mevcut çalışmada, *Usnea longissima* Ach. ve onun sekonder metaboliti usnik asidin insan gastrik adenokarsinom hücreleri (AGS), insan kolorektal adenokarsinom hücreleri (Caco-2) ve fare fibroblastları (NIH/3T3) üzerindeki sitotoksik etkileri incelenmiştir. Bu kapsamda, *U. longissima*'dan metanol ve su ekstraktları Soxhlet ekstraktörü ile elde edilmiştir. Usnik asidin karakterizasyonu fourier dönüşümlü kizilötesi spektroskopisi (FTIR) ile gerçekleştirilmiştir. Ekstraktlar ve metabolitin hücreler üzerindeki sitotoksik aktiviteleri 2,3-bis-(2-metoksi-4-nitro-5-sülfofenil)-2H-tetrazolyum-5-karboksanilid (XTT) analizi ile tespit edilmiştir. Medyan inhibitör konsantrasyonu (IC_{50}) değerleri dikkate alındığında, AGS ve NIH/3T3 hücreleri üzerinde daha fazla etkili olan uygulama metanol ekstraktı olmuştur (sırasıyla, 373.17 µg/ml ve 318.81 µg/ml). Caco-2 hücreleri dikkate alındığında, su ekstraktının en düşük IC_{50} değerine (230.05 µg/ml) sahip olduğu belirlenmiştir. Usnik asidin normal hücreye (NIH/3T3; IC_{50}: 472.41 µg/ml) kıyasla kanserli hücreler (AGS; IC_{50}: 395.03 µg/ml ve Caco-2; IC_{50}: 462.35 µg/ml) üzerindeki yüksek sitotoksik etkinliği dikkat çekmiştir. Sonuç olarak, başta usnik asit olmak üzere *U. longissima*'nın metanol ve su ekstraktlarının tamamlayıcı tedavi kapsamında kullanılabilecek ürünler olduğu ortaya çıkmıştır.

Anahtar Kelimeler: Antikanser, ekstrakt, liken, sekonder metabolit

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1. Introduction

Lichens are unique organisms formed from a symbiotic association between fungi and green algae or cyanobacteria (Ranković and Kosanić, 2019). *Usnea longissima* Ach. is a species with a long and vine-like structure that can have different structures of lichens. It is usually wrapped around tree branches or other surfaces and can sometimes be quite tall (Storaunet et al., 2014; Esseen and Ekström, 2023). This species plays an important role in ecosystems, providing shelter and a food source for organisms living in natural environments. On the other hand, it can also be used as a biological indicator for monitoring air quality (Emsen and Aslan, 2018).

The medical importance of *U. longissima* is related to its use in traditional medicine and some cultures (Rajeswari et al., 2019). In traditional medicine, certain types of lichens are associated with antibacterial properties (Somphong et al., 2023), antifungal (Rajendran et al., 2023b) and anti-inflammatory (Rajendran et al., 2023a) properties. For example, some ancient physicians suggested the use of

lichens in the treatment of wounds and skin diseases (Guo et al., 2008). Some studies have shown that certain compounds in the content of lichens have potential biological activities. For instance, research on the antimicrobial properties of lichens has shown that they can potentially inhibit the growth of bacteria and fungi (Yang et al., 2023). In addition, many studies have been carried out on cytotoxic activities on different cells (Şahin et al., 2021; Ureña-Vacas et al., 2022).

Lichens such as *U. longissima* contain secondary metabolites with many biologically active compounds (Bharti et al., 2022). These compounds are believed to be the defense mechanisms of lichens against environmental stresses. Moreover, it is known that some of these compounds may possess antimicrobial, antiviral, antioxidant, and other pharmacological activities (Adenubi et al., 2022). Usnic acid, the most common secondary metabolite in lichens, is found in *U. longissima* (Divya Reddy et al., 2019). As a chemical structure, usnic acid, a phenolic acid, is a molecule containing a carbohydrate moiety and a phenolic ring. Usnic acid is a compound known to have antimicrobial properties. Its inhibitory effect on bacteria and fungi is considered one of the defense mechanisms of lichens against environmental stresses (Popovici et al., 2022). Therefore, usnic acid may help lichens to gain competitive advantage in their habitat. Research on the antibacterial (Bangalore et al., 2023), antiviral (Miah et al., 2023), antioxidant (Maulidiyah et al., 2023), and anticancer (Emsen et al., 2018) properties of usnic acid has drawn interest for its potential medical uses. In particular, it is thought to be used in traditional medicine to promote wound healing (Stoica Oprea et al., 2023), skin infections, and other health problems (Studzińska-Sroka et al., 2019).

In line with the above-mentioned potential of *U. longissima* and its secondary metabolite, usnic acid, we aimed to examine cytotoxic effects of different extracts of *U. longissima* and usnic acid on human gastric adenocarcinoma cells (AGS), human colorectal adenocarcinoma cells (Caco-2) and mouse fibroblasts (NIH/3T3).

2. Materials and Method

2.1. Collection and Identification of the Lichen Samples

Usnea longissima samples were collected from Oltu district of Erzurum province of Turkey. Samples photographed in their natural environment and were brought to the laboratory and dried. Identification was made with macroscopic and microscopic data using the literature (Purvis et al., 1992; Wirth, 1995). The species was taxonomically identified by Dr. Tubanur Aslan Engin.

2.2. Preparation of the Extracts and Isolation of Secondary Metabolite, Usnic Acid

Lichen specimens were air-dried at room temperature and then powdered. The extraction process involved obtaining methanol (ULME) and water (ULWE) extracts of *U. longissima* using a Soxhlet extraction apparatus with 250 mL solvent systems.

The resulting crude extract from the lichen sample was filtered using a Whatman No. 1 filter paper. The solvent was subsequently removed by evaporation through a rotary evaporator under vacuum conditions, leading to complete drying. The extract was further lyophilized to yield ultra-dry powders. The determination of functional groups within the organic compound structure and bonds present in the dried lichen powders was accomplished using a Bruker Alpha model Fourier Transform Infrared Spectroscopy (FTIR) device spectroscopy.

2.3. Cytotoxic Activity

The XTT method, employing 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, operates on the basis of metabolically active cells converting XTT, a tetrazolium salt, into orange formazan components. The absorbance increase, resulting from color formation, is directly proportional to the number of active cells. An advantageous aspect of the XTT method is its lack of necessity for extra agents or cell washing steps, while maintaining sensitivity even at low cell concentrations (Roehm et al., 1991). This study assessed the 24-hour cytotoxic impact by applying different extract concentrations from lichens on various cell lines, namely AGS, Caco-2, and NIH/3T3. Cells previously stored in liquid nitrogen were thawed, counted, and suspended in DMEM-F12 medium, then transferred to culture flasks and incubated under specified conditions.

For cell passage, trypsin-EDTA-treated cells were suspended, counted, and prepared for cytotoxicity tests. Seeding 10^4 cells/well in sterile 96-well plates allowed adherence and proliferation over 24 hours before applying lichen extracts at varying concentrations for cytotoxicity assessment (n = 3). Lichen extracts were sterilized through filtration before addition to cells, with dilutions prepared in DMEM-F12 medium. Following 24-hour incubation at 37°C in a 5% carbon dioxide incubator, the XTT test was conducted. Extract concentrations ranged from 50 to 1000 µg/ml, with 2% dimethyl sulphoxide (DMSO) serving as the negative control. Extract-containing media was replaced, and wells received 100 µL of 0.5 mg/mL XTT solution with phenazine. Plates were further incubated for 4 hours at 37°C, and absorbance at 450 nm was measured using a multi-plate reader to calculate cell viability as a percentage using the formula: Viability = (Extract absorbance / Control absorbance) × 100.

2.4. Statistical Analyses

The activities of the treatments were analysed one-way ANOVA followed by Duncan test. Probit regression analysis was used to calculate the median inhibitor concentration (IC_{50}) values. Heatmap analysis was utilized to investigate the similarities and dissimilarities of viabilities among the cells. All analyses were done using SPSS (version 21.0, IBM Corporation, Armonk, NY, USA).

3. Results and Discussion

3.1. Characterization of Lichen Samples

FTIR spectrums of lichen sample and usnic acid were presented in Figure 1. The absorption between the range of 3600 and 3100 cm^{-1} can be assigned to the stretching vibrations of the OH groups which might be responsible for the moisture content (İnan and Özçimen, 2021). 3000-2800 corresponded to methylene C-H asymmetric stretching vibration, 1750-1500 and 1200-1000 cm^{-1} which representing C=O stretching functional group and C-O functional group respectively (Bakar et al., 2014). The

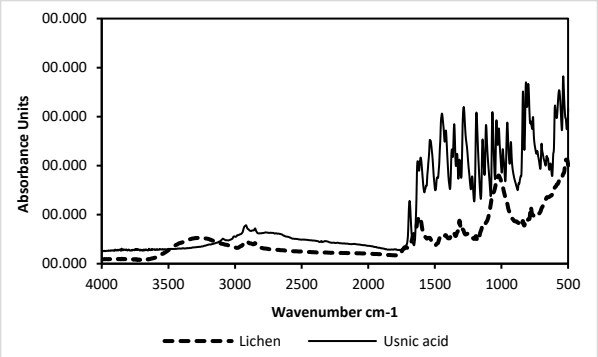

Figure 1. FTIR spectrums of lichen sample and usnic acid

specific bands were observed around 1690 cm^{-1} and 1540 cm^{-1} indicated a non-chelated aromatic ketone and conjugated chelated carbonyl (Karabacak et al., 2014).

3.2. XTT Viability Test

The cytotoxic effect of lichen extracts was examined on AGS, Caco-2, and NIH/3T3 cell lines. The cytotoxic effects of the lichen extracts and usnic acid on the AGS cell lines are presented in Figure 2. It was observed that the anticancer activity of the methanol and water extracts of the lichens increased with increasing concentrations. Moreover, it was found that the methanol extracts of lichen decreased the viability of the AGS cells more than the water extracts. The effect of usnic acid was almost similar to the water extracts of the lichens. The most effective concentration was found to be 1000 µg/ml of methanol extracts of lichen, with a 31% viability of AGS cells. The p-values of the concentrations of 50 and 100 µg/ml of ULME and ULWE were not significant, as can be seen in Figure 2, indicating no effect on the AGS cells. In addition to that, IC_{50} values of ULME, ULWE, and usnic acid were found to be 373.17, 423.21, and 395.03 µg/ml, respectively. According to these values, the most effective application was ULME on AGS cells (Table 1).

As for the Caco-2 cells, cytotoxic activity of the lichen extracts was determined at a concentration of 50 µg/ml (Fig. 3). Similar to the anticancer activity results of the AGS cells, viability of the Caco-2 cells decreased with increasing concentrations. However, the lichen extracts and usnic acid were more effective on Caco-2 cells in comparison with the AGS cells. Cell viability rates at concentrations of 500-1000 µg/ml of ULWE and ULME were not significantly different ($p > 0.05$) from each other. The IC50 values for ULME, ULWE, and usnic acid were found to be 368.02, 230.05, and 462.35 µg/ml, respectively. According to these values, the most effective application was ULWE on Caco-2 cells (Table 1).

In Figure 4, the cytotoxic effect of ULME, ULWE, and usnic acid on NIH/3T3 cells was shown. It was observed that lichen extracts showed a cytotoxic effect on NIH/3T3 cells starting at a concentration of 50 µg/ml. In this analysis, ULME (IC_{50}: 318.81 µg/ml) was found to be the most effective on fibroblast cells in comparison with ULWE (IC_{50}: 367.28 µg/ml) and usnic acid (IC_{50}: 472.41 µg/ml). Between the concentrations of 50-1000 µg/ml, viability of NIH/3T3 cells decreased from 81% to 34% during the experiments. Statistically, the p-values of ULWE and usnic acid at a concentration of 50 µg/ml were not statistically

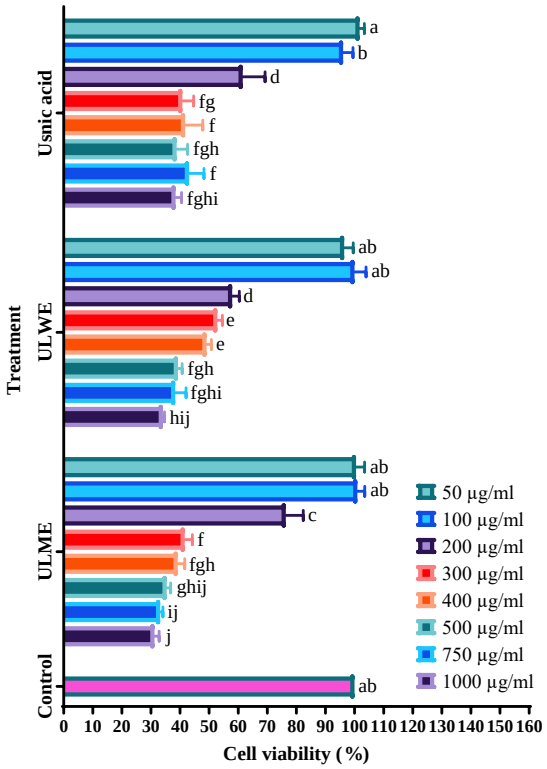

Figure 2. Viability rates obtained by XTT analysis in AGS cells treated with *U. longissima* extracts and its secondary metabolite (Mean ± Standard Deviation, $n = 3$) (Values indicated by different letters differ from each other at the level of $p < 0.05$). ULME: Methanol extract of *U. longissima*; ULWE: Water extract of *U. longissima*.

Table 1. IC_{50} values (µg/ml) resulting from cytotoxic activities of *U. longissima* extracts and its secondary metabolite on different cells

Cell	Treatment	IC50 (Limits)	Slope ± Standard error of the mean (Limits)
AGS	ULME	373.17 (349.35–397.75)	1.18 ± 0.09 (1.16–2.03)
	ULWE	423.21 (392.79–457.03)	1.66 ± 0.07 (1.51–1.81)
	Usnic acid	395.03 (364.87–428.01)	1.49 ± 0.08 (1.32–1.66)
Caco-2	ULME	368.02 (347.41–390.01)	1.96 ± 0.07 (1.82–2.10)
	ULWE	230.05 (207.35–254.06)	1.18 ± 0.06 (1.05–1.30)
	Usnic acid	462.35 (402.44–539.25)	0.82 ± 0.08 (0.66–0.99)
NIH/3T3	ULME	318.81 (286.90–354.88)	0.97 ± 0.05 (0.86–1.09)
	ULWE	367.28 (335.22–404.13)	1.29 ± 0.06 (1.16–1.42)
	Usnic acid	472.41 (412.94–549.49)	0.83 ± 0.06 (0.71–0.95)

ULME: Methanol extract of *U. longissima;* ULWE: Water extract of *U. longissima*

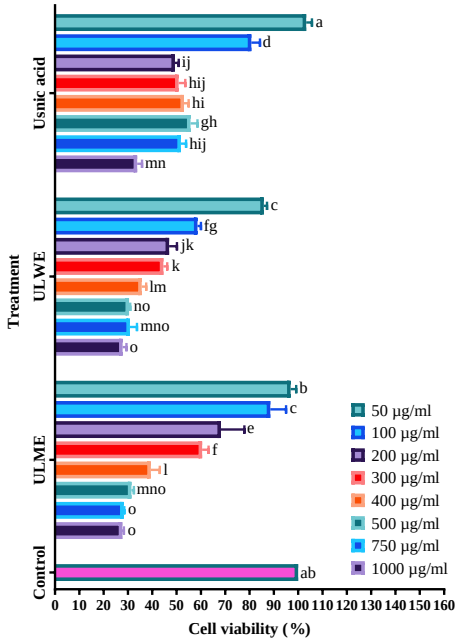

Figure 3. Viability rates obtained by XTT analysis in Caco-2 cells treated with *U. longissima* extracts and its secondary metabolite (Mean ± Standard Deviation, $n = 3$) (Values indicated by different letters differ from each other at the level of $p < 0.05$). ULME: Methanol extract of *U. longissima*; ULWE: Water extract of *U. longissima*.

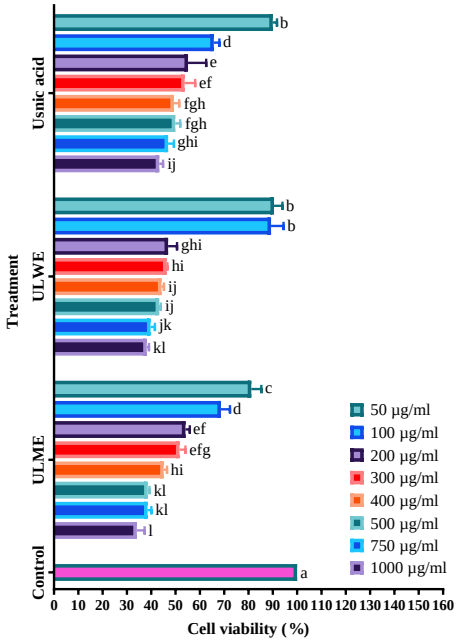

Figure 4. Viability rates obtained by XTT analysis in NIH/3T3 cells treated with *U. longissima* extracts and its secondary metabolite (Mean ± Standard Deviation, $n = 3$) (Values indicated by different letters differ from each other at the level of $p < 0.05$). ULME: Methanol extract of *U. longissima*; ULWE: Water extract of *U. longissima*.

significant. Similarities and differences of different concentrations of ULME, ULWE, and usnic acid on the cells were measured by heatmap analysis. Accordingly, the 50 µg/ml applications of all treatments showed a green gradient on all cells, indicating low cytotoxicity. In addition, the experiments at 500-1000 µg/ml showed high cytotoxicity and were close to each other with the red gradient (Figure 5).

ULME and ULWE exhibited distinct effects on cancer cell lines, with variations likely attributed to factors such as extract concentration, the specific type of cancer cell being tested, and the duration of exposure during cytotoxicity analysis. For example, a study was conducted focusing on eight different lichens and their impact on various cancer cell lines, including L1210 (lymphocytic leukemia), 3LL (Lewis lung carcinoma), K562 (chronic myelogenous leukemia), U251 (glioblastoma), DU145 (prostate carcinoma), and MCF7 (breast adenocarcinoma). The study also included non-cancerous cells represented by the Vero cell line (African green monkey kidney cell line). The results indicated that certain lichens demonstrated heightened activity in terms of cytotoxic effects. Notably, the diethyl ether fractions of *Cladonia convoluta* and *C. rangiformis*, along with the n-hexane fraction of *P. caperata*, exhibited particularly noteworthy anticancer potential (Bézivin et al., 2003).

The researchers conducted a study exploring the impact of methanol extracts from lichens, namely *Parmelia sulcata*, *Flavoparmelia caperata*, Evernia prunastri, *Hypogymnia physodes*, and *Cladonia foliacea*, on colon cancer cells (HCT-116). These extracts were tested across concentrations ranging from 50 to 1000 µg/ml for durations of 24 and 72 hours. The most significant inhibition of cell growth was observed at the highest concentration (1000 µg/mL) after both exposure periods. Notably, extracts from

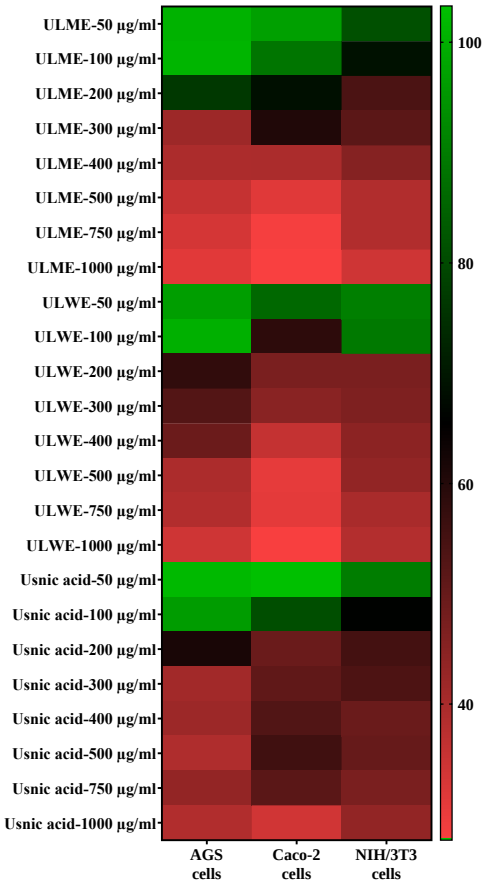

Figure 5. Heatmap of viability rates obtained in the cells treated with different concentrations of *U. longissima* extracts and its secondary metabolite. (High and low activities were represented by green and red colour, respectively). ULME: Methanol extract of *U. longissima*; ULWE: Water extract of *U. longissima*.

Flavoparmelia caperata, *Hypogymnia physodes*, and *Cladonia foliacea* exhibited considerable dose- and time-dependent suppression of cell growth (Mitrović et al., 2011). In another study, cytotoxic properties of lichens gathered from Vietnam National Park were investigated. One extract obtained from *Usnea fammea* Stirt., derived using methanol, demonstrated substantial cytotoxic effects against MCF-7 cells. However, these tested lichen extracts only displayed slight cytotoxicity on fibroblasts at a screening concentration of 100 µg/ml. The lichen extracts exhibited diverse cytotoxic activities against MO-91 cells, but the effective concentrations were over 50 mg/mL (Nguyen et al., 2019). In a separate study, the chemical composition of extracts from *Parmelia conspersa* and *Parmelia perlata* lichens was explored, assessing their antimicrobial, antioxidant, and anticancer attributes. The extracts' cytotoxic effects on RD, Hep2c, and L2OB cells ranged from 76.33 to 163.39 µg/mL (Manojlović et al., 2021). Lichen compounds induce cell death through various molecular mechanisms, including cell cycle arrest, apoptosis, necrosis, and inhibition of angiogenesis. Brisdelli et al. highlighted these mechanisms as the primary pathways through which lichen compounds exert their lethal effects (Brisdelli et al., 2013). The researchers emphasized that lichen compounds have been scientifically proven to possess anticancer properties, reinforcing their potential in combatting cancer cells (Manojlović et al., 2021).

Conflict of Interest

There is no conflict of interest in any form between the authors.

Authors' Contributions

The authors contributed equally.

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