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In vitro Cytotoxic Activity of a Sulphated Polysaccharide Ulvan against Human Breast and Glioblastoma Cell line

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ABSTRACT: This study was designed to evaluate the hemolysis, anti-inflammatory and cytotoxicity activity of sulphated polysaccharide ulvan extracted from green seaweed *Ulva lactuca* (UL). Ulvan was extracted and characterized with UV-Visible Spectroscopy, Fourier transform infrared spectroscopy (FTIR) and Scanning electron microscopy (SEM). Total uronic acid and sulphate content were found to be 19.38% w/w and 22.53% w/w. The biocompatibility of the sulphated polysaccharide ulvan was evaluated via hemolysis, its interaction with erythrocyte structure and anti-inflammatory studies. Further, the polysaccharide was treated for cellular toxicity against human breast cancer (MCF-7) and human glioblastoma cancer (U87) cell lines using the 3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay method. This study indicates that the sulphated polysaccharide ulvan was more suitable for biomedical applications with promising chemo preventive and chemotherapeutic activity.

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INTRODUCTION

In recent years, various marine resources have been considered for the search of bioactive compounds to develop new drugs and health food. Seaweed polysaccharides are highly active natural substances having beneficial properties. However, among the three main divisions of seaweed: green seaweed (Chlorophyta), brown seaweed (Phaeophyta) and red seaweed (Rhodophyta), where the use of green seaweed remains largely unexploited.

Ulva lactua, also known by the common name "sea lettuce" is edible green seaweed. Ulvan is gaining a tremendous increase due to its beneficial effects provided by the bioactive compounds constituting it [1]. Most of the biological activity exhibited by Ulva has been found to be linked to its content of sulphated polysaccharide commonly labeled as ulvan [2]. Ulvan is a natural water soluble sulphated polysaccharide isolated from green seaweed *Ulva lactua*. Ulvan more specifically contains xylose, glucuronic acid or iduronic acid and rhamnose groups [3,4],Which were proven to occur mainly in the form of aldobiouronic acids, comprising the major repeating disaccharides in ulvan (**Figure 1**) the two different types of aldobiouronic acid were named ulvanobiuronic acid 3-sulphate type A (A_{3s}) and type B (B_{3s}) [5]. The disaccharide A_{3s} is composed of glucuronic acid and sulphated rhamnose, while the disaccharide type B_{3s} consists of iduronic acid and sulphated rhamnose, mainly associated via (1→4) glycosidic bond.

It possesses wide range of pharmacological activities such as anticancer [6], anticoagulant, anti-inflammatory, antihyperlipidemic, antioxidant, antiviral, antipeptic, anti tumor and antiadhesive properties [7, 4]. Ulvan could represent a promising platform of materials suitable for different applications comprising the biomedical field.

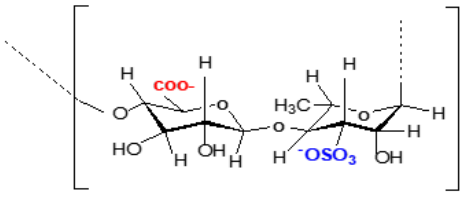

Figure 1 Structure of Ulvan

Cancer is considered as one of the most terrified diseases and it's a class of diseases or disorders characterized by uncontrolled rapid cell proliferation and metastasis of abnormal cell in the body. Breast cancer is the second leading cause of mortality among women [8] and Glioblastoma is the most common intracranial malignancy which constitutes about 50% of all gliomas. Malignant gliomas are the third leading cause of cancer death, accounting for 2.5% of the global cancer death toll [9]. While it can be treated with chemotherapy and surgical applications, the disease can easily relapse. Hence, there is a great interest in the development of safe, low-cost anti-cancer agents from natural sources. Similarly MCF-7 breast cancer cells and U87 glioblastoma cells serves as an excellent source of *in vitro* model for studying the mechanism of tumor response as well as complex relationships between binding and biological systems.

On the basis of these observations, the purpose of the present study was to extract sulfated polysaccharide ulvan from green seaweed *Ulva lactua* and was fully characterized by spectral analysis, surface morphology and anti-inflammatory, hemolysis and its interaction with erythrocyte have been evaluated and also we investigated the cytotoxicity of the ulvan against two human cancer cell lines including human breast cancer (MCF-7) and human glioblastoma (U87) for the selection of potential novel drug for cancer therapy.

MATERIALS AND METHODS

Materials

MCF-7 and U 87 cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India. The 10% bovine serum, Dulbecco's minimal eagles medium (DMEM), antibiotics were purchased from HiMedia, India. All other solvents and chemicals wee of analytical grade.

Collection and Identification of Seaweed

The green seaweed *Ulva lactua* was collected from Mandapam in the Gulf of Mannar and was identified and authenticated by Dr. M. Ganesan, Scientist of Marine Algal Research station, CSMCRI (Central Salt & Marine chemicals Research Institute) Mandapam Camp, Tamil Nadu, India. The collected seaweed was washed with tap water in order to remove the sand particles and epiphytes. Then it was further washed thoroughly with double distilled water, and then dried by air in shade. Once dried the seaweed was crushed and pulverized into powder form, and then stored at room temperature for further use.

Extraction of Ulvan

Ulvan was extracted by the following method [10]. Briefly 30 grams of dried green seaweed powder was placed in 900ml of water. The solution was continuously stirred and kept at 90-95°C in a hot water bath for 2 h. The aqueous extract was centrifuged at 10,000 rpm at 4°C for 15 min. The supernatant was collected and allowed to cool and then precipitated with 4 vol. absolute ethanol. The mixture was kept overnight and precipitate formed was collected by centrifugation and were lyophilized using a freeze dryer (CHRIST Alpha 1–4 LSC basic, Germany). The dried samples were stored in vials at 4°C and used for further analysis. Yield of a sulphated polysaccharide (ulvan) resulting from this extraction methodology is approximately 10 – 20%.

Chemical Characterization

The total sulphate content of the ulvan was determined according to the turbidometric method [11] described by Dodgson and Price, using barium chloride- gelatin. The total uronic acid content of ulvan was also determined by m-hydroxydiphenylmethod [12] using D-glucuronic acid as standard respectively.

UV-Visible Spectroscopy Analysis

UV-Visible absorption spectra for the sample was scanned in the wavelength ranging from 200-800nm by using Perkin Elmer Lambda 35 model.

Fourier Transform Infrared Spectroscopy FTIR

FT-IR spectra of the solid sample in the form of KBR pellets were recorded on Perkin Elmer Spectrum Two FTIR spectrophotometer in the range of 400-4000 cm^{-1} which were used to detect the characteristic peaks and functional groups

Surface Morphology

The morphology of ulvan was determined by scanning electron microscope (Carel Zeiss Evo18).

Biological Characterization

Hemolysis Activity

Fresh human blood sample (3ml) was collected and stored in anticoagulant ethylenediamine tetra acetic acid (EDTA) coated tubes. The blood thus collected was mixed with phosphate buffered saline and subjected to centrifugation for 5 min at 500g.Red blood cells were obtained as a pellet and that was resuspended in 20 ml of PBS. 100µl of diluted RBCs suspension are treated with equal volume of ulvan extract at concentrations ranging from 20 to 100µg/ml. The samples were then incubated at 37°C for 30 min. After incubation the mixture was centrifuged at 500g for 10 min and the absorbance was measured at 540nm using a microplate reader (BioTek Epoch Microplate Reader).RBCs suspended in Triton X 100 and PBS was used as positive and negative control. The results were supported with the microscopic observations of RBCs with ulvan extract [13, 14]. The percentage of hemolysis was calculated using the formula

$$\text{Hemolysis (\%)} = 100 * \frac{(\text{OD sample} - \text{OD negative Control})}{(\text{OD positive control} - \text{OD negative control})}$$

Anti-inflammatory Assay

Membrane Stabilization Activity

Anti-inflammatory activity was conducted by HRBC membrane stabilization method [15]. Briefly, 2 ml of human blood was collected and mixed with equal volumes of Alsever's solution and subjected to centrifugation at 3000 rpm for 10 min. The RBC pellet was further washed with isosaline and suspension was made. Different concentrations of ulvan

was prepared to which 0.5 ml of human red blood cell (HRBC) suspension was added along with 2 ml of hyposaline and 1 ml of PBS and subjected to an incubation of 30 min at 37°C. The suspension was then centrifuged at 3000 rpm for 5 min and measured at 560nm using Microplate reader (BioTek Epoch Microplate Reader). Diclofenac sodium salt served as standard.

Anti-inflammatory activity was further calculated by
%Stabilization = 100 − [(OD $_{sample}$/OD $_{control}$)×100

Cytotoxicity activity

Cell line and culture

MCF-7 and U87 cell line were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

MTT Assay

MTT assay was used to evaluate the cytotoxicity of ulvan on MCF-7 and U87 cells. Briefly 1 X 10^5 cells were seeded well in 24-well tissue culture plates and incubated in 37^0C with 5% CO_2 condition. The cells were then treated with 20 to 100 µg/mL of ulvan for 24, 48, and 72 h respectively. After each exposure the cells were washed twice with PBS and incubated with MTT solution (0.5 mg/mL) for 4 h. After incubation, DMSO was added to each well and the optical density (OD) was read at 570 nm using micro plate reader (Epoch BioTek) .The % cell viability was calculated using the following formula[16]

% Cell viability = A570 of treated cells / A570 of control cells × 100

RESULTS AND DISCUSSION

Chemical Characterization

Ulvan from U*lva lactua*, a sulphated polysaccharide mainly composed of uronic acid, sulphate, rhamnose and trace amounts of sugar. Ulvan extracted from *Ulva lactua* yielded 10-20% contained 22.53%w/w and 19.38%w/w of sulphates and uronic acids respectively.

UV- Visible Spectroscopy

UV spectra of Ulvan show a absorbance peak at around 266.3nm (**Figure 2**).This is attributed to the formation of carbonyl groups or double bond in pyranose ring [17].

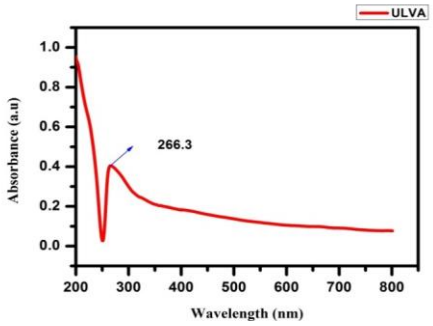

Figure 2 UV Spectrum of Ulvan (*Ulva lactua*)

FT IR Spectroscopy

In order to further characterize ulvan extracted from green algae and to identify the fundamental groups present in its structure, FT-IR analysis was performed. The infrared spectrum of this polysaccharide ulvan with different intensities is given in **Figure 3**.

Ulvan gives a strong band and intense signal within the range (3500cm^{-1}-3300cm^{-1}) which attributes to the stretching vibration of the polymeric hydroxyl group (O-H) characterized to the disaccharide structures that existed in the H-bonds of the molecules. The presence of carboxylate group C=O of uronic acid in ulvan shows a strong bending vibration at 1633cm-^{1} and symmetric stretching band at 1402cm^{-1}[18,19].The asymmetric stretching of the ether glycosidic bridge (CH–O–CH) is shown by an intense band around 1183 cm$^{–1}$. Stretching of ether sulfate groups (RO–SO_3) is represented by a band at about 1265 cm$^{–1}$ [20]. Peak at 847cm-1 might correspond to the bending vibration of C-O-S of sulphate in equatorial position and are related to sugar cycles

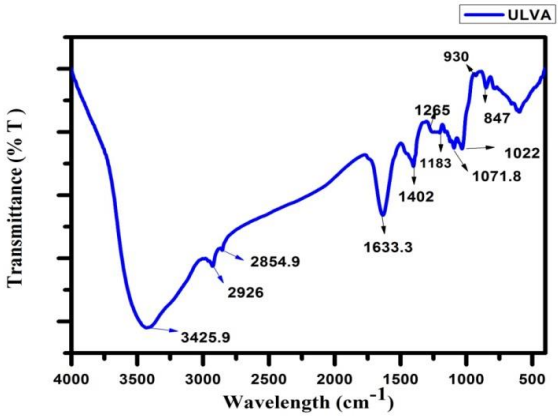

Figure 3 FT IR Spectra of Ulvan

Scanning Electron Microscopy

Scanning electron microscope (SEM) was carried out to find the surface morphology of Ulvan. Structure of the ulvan seems to be rod shaped with a smooth surface. It is notable that the particle seems to merge together and become embedded with the adjacent particles. In addition, much more agglomerated structure can be seen (**Figure 4**)

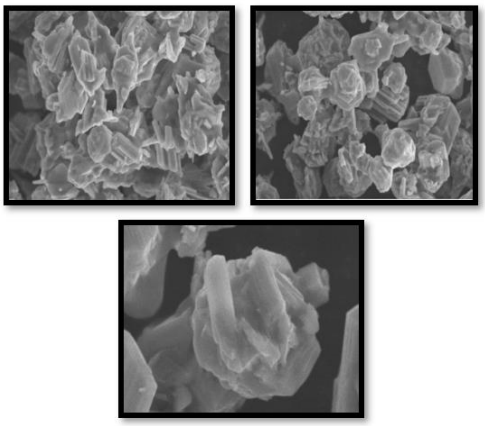

Figure 4 SEM images of Ulvan

Biological Characterization

Hemolysis Activity

The *in vitro* biocompatibility analysis of ulvan is a vital factor for its biomedical applications. The RBC membrane was incubated with different concentrations (20-100µg/ml) of ulvan. The negatively charged oxygen present on ulvan electrostatically interacts with the positively charged phosphatidylcholine lipids present on outer surface of red blood cells thus contributing to the hemolytic activity of ulvan. At a concentration of 100µg/ml the percentage hemolysis was recorded to be 12.38(**Figure 5**). The data was further supported with light microscopy images of RBC (**Figure 6**).

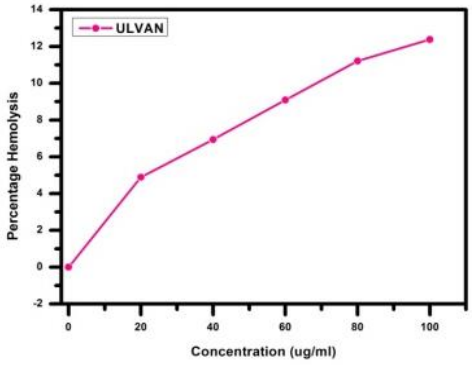

Figure 5 Percentage Hemolysis Activity of Ulvan

Anti-inflammatory Activity

One of the most important criteria for a material to be biocompatible is it shouldn't cause any kind of inflammation. The red blood cells are considered as an analogue of lysosomal membranes thus stabilization of RBC by hypotonic solution is considered as an in vitro measure of anti-inflammatory activity. With increase in concentration the percentage stabilization increased (**Figure 7**). At 100µg/ml, the percentage of stabilization was approximately 90% for ulvan.

Cytotoxicity Analysis

The MTT assay was studied to determine the cytotoxicity of the ulvan extract against MCF-7 and U87 cell lines. The results give in **Figure 8A, 8B**, and **Table 1** shows the cytotoxicity of treatments examined at different concentrations (20 to 100µg/ml). Cell viability was markedly decreased with different concentrations of extract. The growths of cells were inhibited in a concentration and time dependent manner.

The green marine algae ulvan showed a stronger selective cell proliferation inhibition of the cancer cell line. After 72h of incubation ulvan showed a maximum inhibition of 90% at a concentration of 100µg/ml in U87 cell line, whereas a maximum inhibition of 78.2% was observed at the same concentration in MCF-7 cell line. From the results it clearly indicates that the ulvan extracted from *Ulva lactua* showed a significant cytotoxicity activity on both MCF-7 & U87 cell lines. The data's were further supported with morphological images as shown in **Figure 9 & 10**.

Extract	Cell line	Concentration (µg/ml)	Cytotoxicity (%)			Cell Viability (%)		
			24h	48h	72h	24h	48h	72h
Ulvan	MCF-7	20	19.1	32.5	62.85	80.99	67.4	37.15
		40	25.97	37.87	66.8	74.03	62.13	33.2
		60	32.85	43.36	70.63	67.15	56.64	29.37
		80	40.25	48.78	74.65	59.75	51.22	25.35
		100	47.12	54.13	78.22	52.88	45.87	21.78
	U87	20	40.67	58.35	64.69	59.33	41.65	35.31
		40	47.09	63.75	70.95	52.91	36.25	29.05
		60	53.12	69.14	77.05	46.85	30.86	22.95
		80	60.12	74.37	83.15	39.88	25.63	16.85
		100	66.54	79.6	90	33.4	20.4	10.0

Table 1 Cytotoxicity Activity of *Ulvan* on MCF-7 & U87 cell line using MTT Assay

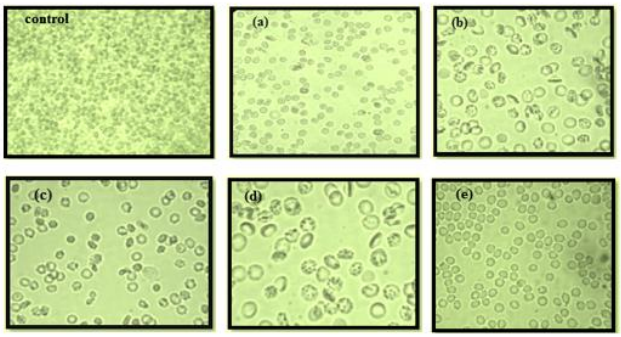

Figure 6 Optical Microscopic images of RBC on treatment with ulvan at different concentrations Control (a) 20µg/ml (b) 40µg/ml (c) 60µg/ml (d) 80µg/ml (e) 100µg/ml

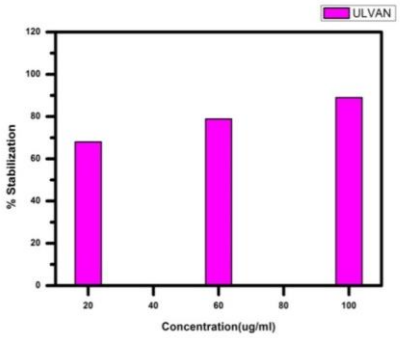

Figure 7 Percentage Stabilization of Ulvan

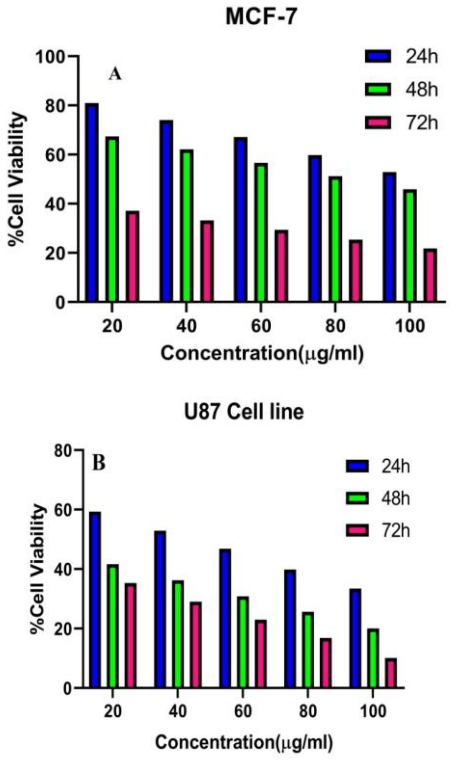

Figure 8A & 8B –Cytotoxicity Evaluation of Ulvan at various concentrations on MCF-7 Cell line and U87 Cell line

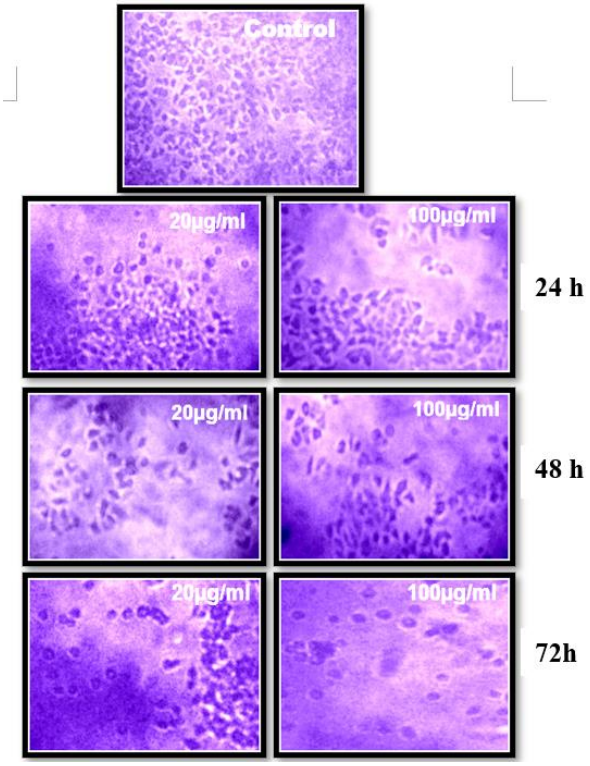

Figure 9 Morphological observation of MCF-7 control and treated cells under inverted microscope

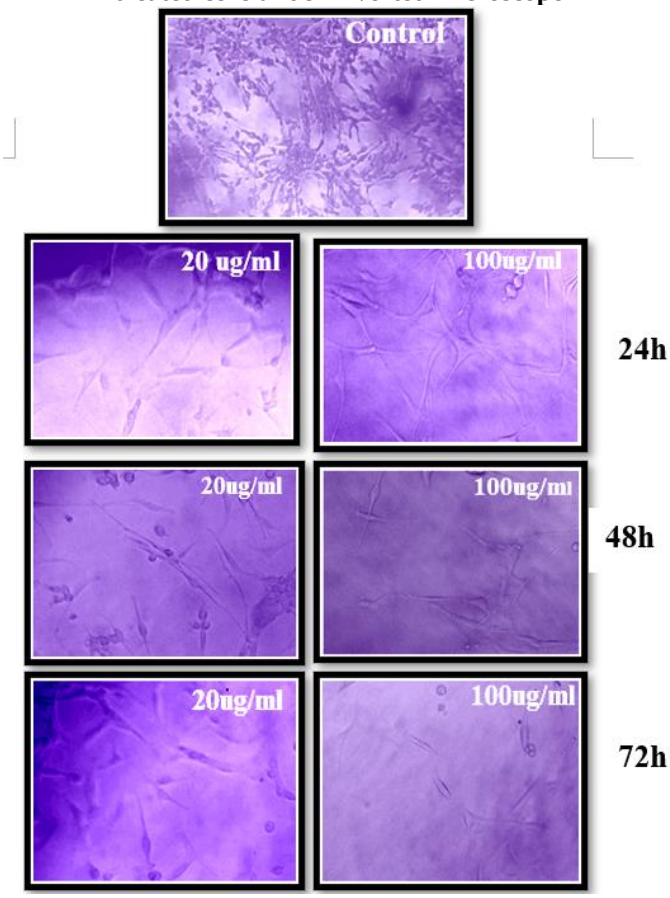

Figure 10 Morphological observation of U87 control and treated cells under inverted microscope

CONCLUSION

The results of the preset work indicated that the aqueous extract of Ulvan, a sulphated marine polysaccharide from green marine algae *Ulvan lactua* is of great versatility, non-toxic and eco friendly. The significant biological activities of ulvan, in combination with its tunable physicochemical properties have triggered high interest for its utilization in hybrid biomaterials. The hemolytic and inflammatory activities of naive ulvan make it more biocompatible. The ulvan showed a significant cytotoxic activity against human glioblastoma and human breast cancer cell line in a concentration and time dependent manner and it can be developed as a promising cancer fighting compound for various types of cancer. Marine polysaccharide based nanomaterials have a great promise in biomedicine, fabric, food, and pharmaceutical industries for the future. Further research is needed to isolate and characterize the components responsible for their biological activities.

DATA AVAILABILITY

Not declared

ETHICS STATEMENT

The authors have taken all the necessary permissions as per ethical guidelines wherever applicable. The authors will be responsible for all the technical content mentioned in the manuscript. Journal and Publisher will not be responsible for any copyright infringement and plagiarism issue.

CONFLICTS OF INTEREST

The authors have no known conflict of interest.

FUNDING INFORMATION

Not declared.

AUTHORS' CONTRIBUTION

All the authors were contributed for manuscript preparation, conducting of the experiments, data collection, interpretation and analysis of the result.

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