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Preliminary Evaluation of Cyclooxygenase-2 Inhibition in Solvent Extracts of Leaves of Simarouba glauca. An In Vitro Study.

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Abstract: The study aimed to determine the anti-inflammatory activity through cyclooxygenase-2 inhibition in the solvent extracts of Simarouba glauca leaves. The Soxhlet extraction of dried leaves was carried out with methanol, ethanol, chloroform and water. The anti-inflammatory activity in ethanol and methanol extracts of leaves was studied by protein denaturation inhibition and the Cyclooxygenase-2 expression in THP-1 monocytic cell line by flow cytometry with diclofenac and aspirin as the standard drugs respectively. A concentration dependent inhibition of albumin denaturation by the solvent extracts of S glauca leaves was observed. The ethanol extract showed twice the protein denaturation inhibition, while the methanol extract was almost same as the standard (75.95%) at 500µg/ml. The cyclooxygenase-2 expression was induced using lipopolysaccharide (LPS). The geometric mean fluorescence intensity (MFI) due to addition of the solvent extracts was measured. The ethanol and methanol extracts expressed geometric mean cell count of 60488 and 126501 respectively. The study showed that the ethanol extract of leaves has the potential to the decrease the inflammation compared to the methanol extracts. The anti-inflammatory activity is due to the phytochemicals present in the leaves. Further studies are essential to ascertain the use of the plant for preparation of anti-inflammatory drugs.

Keywords: Simarouba glauca. phytochemicals, anti-inflammatory, cyclooxygenase-2, flow cytometry.

1. INTRODUCTION

Cyclooxygenase (COX), prostaglandin endoperoxide synthetase (EC.1.14.99.1) is an enzyme involved in the metabolism of arachidonic acid and biosynthesis of prostanoid, proinflammatory prostaglandins (PGE_2, $PGF_{2\alpha}$) [1]. COX occurs in two isoforms COX-1 and COX-2. COX-2 is expressed in tissue damage or inflammation because of proinflammatory cytokines namely, TNF-α, IL-1β and interferon gamma [2]. Inflammation is a natural defense mechanism designed in the organisms to offer protection against infections due to invasion of foreign bodies and any injury. Inflammation is characterized by edema, pain, fever and burning sensation. It is classified as acute and chronic. The acute form persists for a short period, while the chronic persists for longer period. The chronic inflammation is dependent up on the inflammatory mediators produced in the affected area [3]. Sometimes inflammation can be harmful to the organism and contribute to number of diseases. Immune system is also linked to the formation of reactive species by the activated macrophages and neutrophils. The cells secrete mediators like the cytotoxic reaction intermediates, prostaglandins etc. [4]. Researchers have suggested that over countenance of cyclooxygenase-2 is responsible for inflammatory diseases such as Helicobacter pylori associated gastritis, Crohn's disease, ulcerative colitis, cardiovascular disease and rheumatoid arthritis [5]. Administration of NSAIDs for reducing the inflammatory condition is very common. The use of traditional medicine from plants is being substituted due to many adverse effects caused by the synthetic drugs. Studies have demonstrated the use of plant based natural products can modulate proinflammatory molecules like the cytokines and cyclooxygenase-2 which are the key factors for inflammation [6].
Simarouba glauca, an evergreen tree belongs to Simaroubaceae family and the specific name glauca, is taken from the Greek word meaning blossom which means bluish green foliage. The significant bioactive groups of chemicals that have been reported in Simarouba are the quassinoids [7]. In several nations it is considered as medicinal plant in due to

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the various health benefits and are safe to use with no serious side effects. It has currently created great enthusiasm as miraculous tree of solace for cancer patients [8-9].

2. MATERIALS AND METHODS

The leaves of S. glauca were collected from GKVK, University of Agricultural Sciences, Bengaluru. Authentication of the plant was done at Regional Ayurveda Research Institute for Metabolic disorders, Ref no. RRCBI-mus215. The leaves were washed thoroughly and dried in shade for 30–40 days at room temperature. The analysis was carried out after the dried leaves were pulverized to a powder.

Fig. 1. Simarouba glauca- leaves

2.1 Sample Preparation

30g of S. glauca dried leaves powder was taken and subjected to the successive solvent extraction process in the order of their polarity using a Soxhlet apparatus. Chloroform, ethanol, methanol, and water were taken for the solvent extraction. The extraction was done with 250 ml of each solvent separately. The process was time framed for complete 48 h, after which the solvent mixture was concentrated using a rotary vacuum evaporator [10,11]. The sample extracts obtained were placed in moisture free condition and used to study the phytochemical analysis, antioxidant, anti-inflammatory and cyclooxygenase activity.

2.2 Egg Albumin Denaturation Assay

Egg albumin (hen's egg from local market in Bangalore), phosphate buffered saline (PBS) pH 6.4 (Merck, India), diclofenac sodium (Symed Pharm. Pvt, Ltd, Hyderabad), BOD incubator (Royal Scientific, India) and spectrophotometer (UV-1800, Shimadzu, Japan).

0.2 ml egg albumin (fresh) and 2.8 ml of PBS was mixed to 2 ml of varying samples of S glauca extracts. The plant extracts were prepared at three different concentrations, 100 µg/ml, 250 µg/ml and 500 µg/ml. 5 ml double-distilled water served as control. Later, reaction mixtures were placed at 37 ℃ in a BOD incubator for 15 min, subsequently warm up for five minutes. This was allowed to cool, and absorbance was read at 660 nm [12]. Diclofenac sodium as positive control was considered for the assay. The procedure performed was in triplicates. The percentage inhibition was calculated from the mean absorbance, using the formula:

Percentage inhibition = 100 × (Vt / Vc - 1)

Where, Vt = absorbance of test sample or the standard, Vc = absorbance of control.

2.3 Cyclooxygenase-2 Expression (COX 2) Analysis by Flow Cytometry

Phosphate-buffered saline (PBS), Lipopolysaccharides (LPS), Trypsin-EDTA solution, 2% paraformaldehyde solution, 0.5% BSA in 1X DPBS, 0.1% Triton-X 100 (Hi Media, India) in 0.5% BSA solution (HiMedia, India), 15 ml and 1.5 ml centrifuge tubes (Abdos, India), aspirin Symed Pharm. Pvt, Ltd, Hyderabad). PE Mouse Anti-Human COX-2 Catalog No. 565125, BD GolgiStop™, Protein Transport Inhibitor (Containing Monensin), Catalog No. 554724, Flow cytometer: BD Accuri C5 Flow cytometer (BD Biosciences) and analysis software: Flow Jo v10.0.7.

Cyclooxygenase 2 (COX 2) expression was determined using THP-1 monocytic cell line. The cell culturing was carried out in a 96-well plate at a density of 3 x 10^5 cells/2 ml and placed in a CO_2 incubator at 37°C for 24 hours. The cells (in all the wells except the Untreated) were stimulated with lipopolysaccharide (LPS) (2 µg/ml) 4 hours prior to plant extract treatment. 1 µl of BD GolgiStop™ was transferred to all wells for the accumulation of expressed proteins within each cell. The cells were treated with required concentration of Simarouba glauca samples (50 µg/ml) and

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standard (aspirin – 25 µg/ml), incubated for 6 hours. Later, the cells were garnered to 15 ml centrifuge tubes and gyrated for 5 min at 300 x g at 25°C. The supernatant was removed cautiously and rinsed with PBS for a couple of time. 0.5 mL 2% paraformaldehyde solution was added to all tubes and stored for 20 minutes, washed using 0.5% bovine serum albumin (BSA) in 1X PBS. Then 0.1% Triton-X 100 in 0.5% BSA solution was added and kept for 10 min after which tubes were again washed with 0.5% bovine serum albumin (BSA) in 1X PBS. Diluted antibody was mixed to 100 µl of cell suspension, stirred thoroughly by pipetting and stored for 30 minutes in the dark at 25°C. Finally, 0.5% BSA and 0.5 ml of PBS was added and mixed thoroughly and transferred to 1.5 ml tubes and analyzed using FL-2 detector for the fluorochrome phycoerythrin (PE) [5].

3. RESULTS AND DISCUSSION

Any damage caused in tissues due to injury is stimulated with enzymatic reactions. The membrane cohesion is responsible for the cellular vitality. When the red blood cells are subjected to hypotonic medium the membrane lysis occur along with hemolysis followed by oxidation of hemoglobin. Consequently, the cell is ruptured due to fluid accumulation. This process leads to lipid peroxidation through free radical generation and results in secondary damage . Molecules that can stabilize the membrane and prevent cell lysis possess the property to interfere the trigger of inflammatory mediators [13].

3.1 Evaluation of protein denaturation inhibition.

The ethanol and methanol extracts of S glauca were evaluated for inhibition of egg albumin denaturation (Fig. 2). The denaturation assay was carried out at 100, 250 and 500 µg/ml concentrations. Diclofenac sodium was used as positive control (standard) for the assay. Results are represented in table1. The percentage inhibition was dose dependent. The ethanolic extract showed almost similar inhibition (73.25%) as the standard (75.94 %) at 250 µg/ml concentration. The methanolic fraction displayed same percentage of inhibition as the standard, while the ethanolic extract expressed twice the inhibition activity from the standard at 500 µg/ml concentration. The in vitro anti-inflammatory action of the ethanol extract of leaves was evaluated. The study reported that percentage inhibition was directly dependent on the concentration of sample taken for the experiment and the ethanolic extract of leaves produced an effective activity of 74% at 1000 µg/ml concentration. Thus, the extract reduced the formation of auto antigens and prevented the denaturation of protein [14]. Anti-inflammatory activity of methanol extracts of S glauca leaves has been reported in the inflammation induced rats and suggested that the leaves demonstrate impressive anti-inflammatory activity [15]. Tissue protein denaturation is documented in inflammatory and arthritic diseases. In vivo studies have revealed the involvement of auto antigens in certain arthritic diseases [16,17]. Molecules with the possibility to prevent protein denaturation are worthwhile for anti-inflammatory drug development. Plant substances from coffee beans, curcumin and other medicinal plants are potential sources for the alleviation of inflammatory diseases [18, 19].

Table.1 Inhibition of egg albumin denaturation by S glauca extracts

Sample	**Percentage inhibition**			
Concentration (µg/ml)	**100**	**250**	**500**	**IC50 (µg/ml)**
Diclofenac	16.25	41.75	75.94	322.6
Ethanol extract	18.7	73.25	143.55	175.4
Methanol extract	16.2	52.31	75.94	306.2

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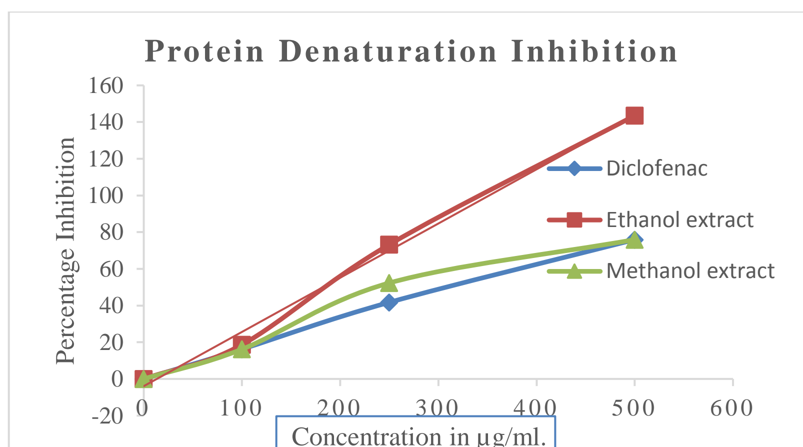
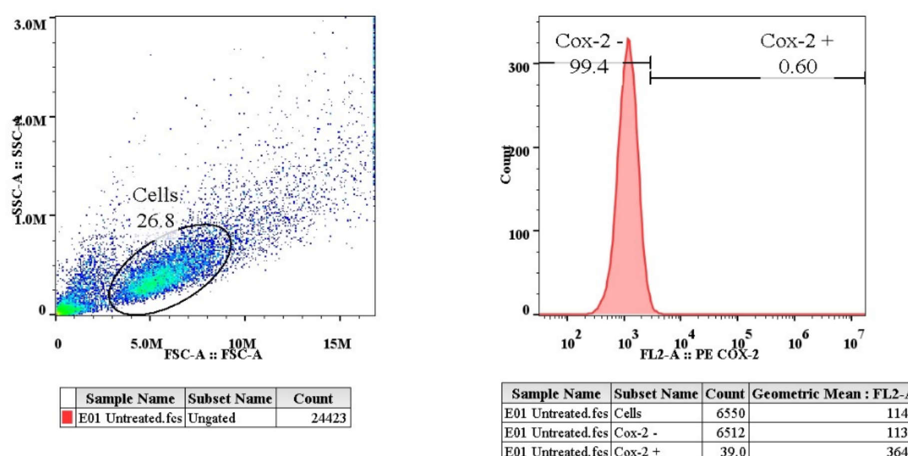


Fig. 2. Inhibition of egg albumin denaturation by ethanol and methanol extracts of S glauca

3.3 COX expression in THP 1 cell lines using S glauca extracts

In the physiological condition COX-2 expression is undetectable but can be induced by several stimuli [20]. Aspirin and other (NSAIDs) alleviate inflammation, swelling, pain; fever and thrombosis by the expression of COX 2 [18] inhibition. COX-2 expression was examined in THP-1 monocytic cell line using flow cytometry and the geometric MFI of PE-A was measured. The ethanol and methanol extracts of leaves were considered for the evaluation of COX-2 expression in THP-1 monocytic cell line, induced by LPS. The negative control cells displayed geometric MFI of 1144 (fig. 3). LPS induced cells showed an increase in the enzyme expression with MFI with 215070 (fig. 4). COX-2 expression in cells treated with aspirin (Standard), post LPS treatment diminished with decrease in MFI (8954) (fig. 5), whereas cells treated with ethanolic extract of leaves showed geometric MFI (60488) and methanol extract (126501) (fig. 6 and 7 respectively). The observation substantiated the results obtained from egg albumin denaturation assay, which showed higher percentage of inhibition in the ethanol extract compared to the methanol extract. Phytochemicals like flavonoids, alkaloids, terpenoids, saponins have proved to possess anti-inflammatory activity [21]. Many studies conducted on the phytochemical analysis of S glauca have reported the presence of these biomolecules, which are produced by the plant as secondary metabolites [11,22-24]. Several reports have documented the COX-2 inhibition by plant extracts are due to the presence of flavonoids, which can reduce the inflammatory disorders [25]. The serious side effects such as cardiovascular malfunction caused with the intake of several highly effective anti-inflammatory drugs (Celecoxib, Valdecoxib etc.) persuaded the pharma industries to withdraw the drugs from the market [26-27].



Sample Name	Subset Name	Count
E01 Untreated.fcs	Ungated	24423

Sample Name	Subset Name	Count	Geometric Mean : FL2-A
E01 Untreated.fcs	Cells	6550	1144
E01 Untreated.fcs	Cox-2 -	6512	1136
E01 Untreated.fcs	Cox-2 +	39.0	3646

Fig.3. COX-2 expression in THP 1 cells-untreated

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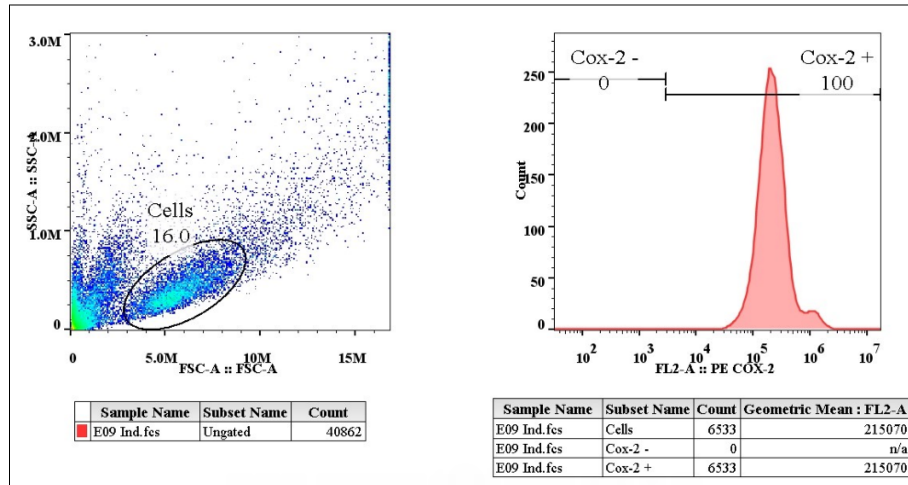
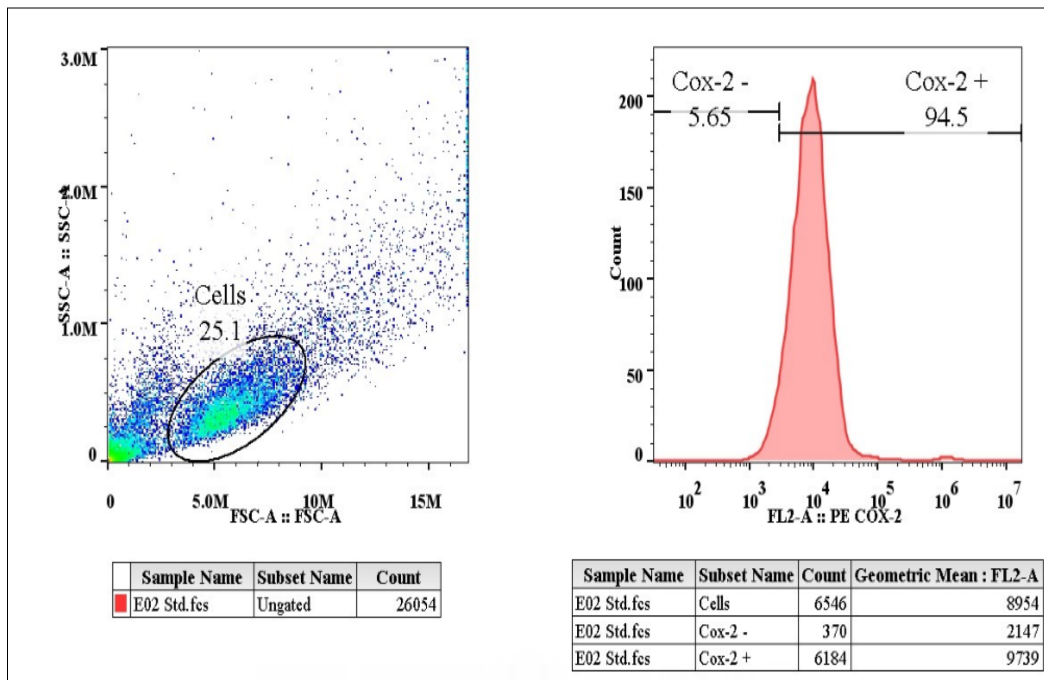


	Sample Name	Subset Name	Count
	E09 Ind.fcs	Ungated	40862

Sample Name	Subset Name	Count	Geometric Mean : FL2-A
E09 Ind.fcs	Cells	6533	215070
E09 Ind.fcs	Cox-2 -	0	n/a
E09 Ind.fcs	Cox-2 +	6533	215070

Fig. 4. COX- 2 expression in LPS induced THP 1 cells.



	Sample Name	Subset Name	Count
	E02 Std.fcs	Ungated	26054

Sample Name	Subset Name	Count	Geometric Mean : FL2-A
E02 Std.fcs	Cells	6546	8954
E02 Std.fcs	Cox-2 -	370	2147
E02 Std.fcs	Cox-2 +	6184	9739

Fig. 5. COX-2 expression in THP 1 cells-aspirin treated

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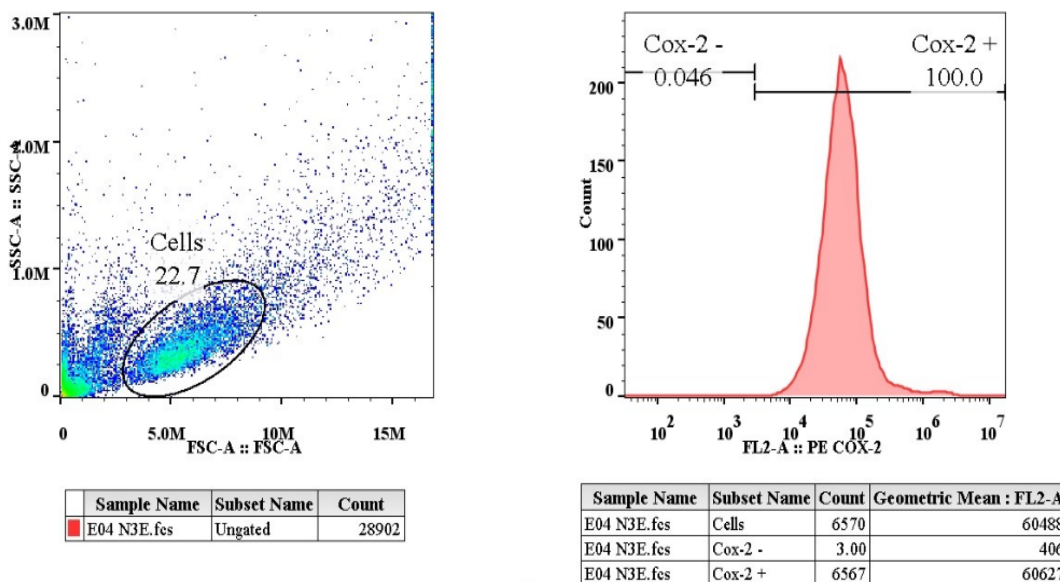

Fig.6. COX-2 expression in THP 1 cells-treated with ethanol extracts of S glauca leaves

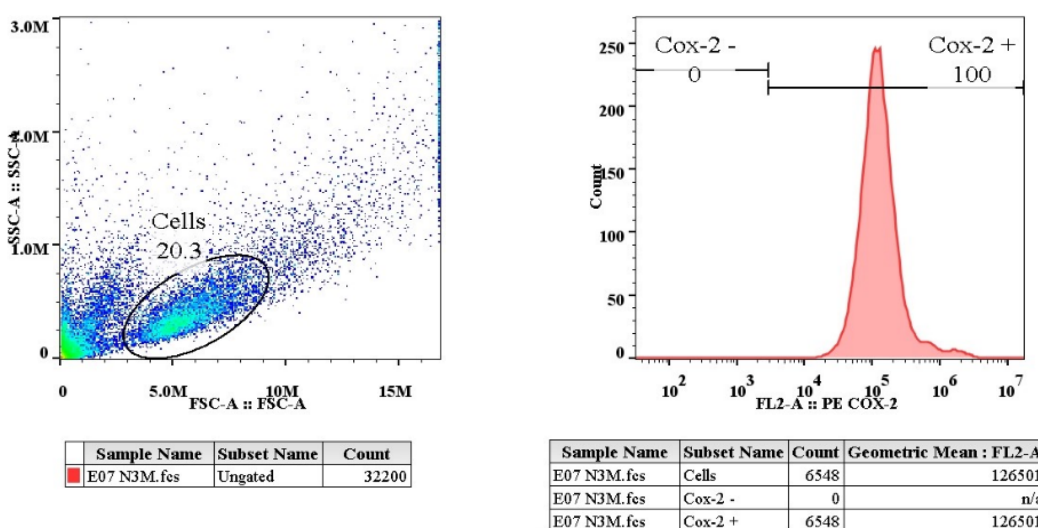

Fig.7. COX-2 expression in THP 1 cells-treated with methanol extracts of S glauca leaves

4. CONCLUSION

An increasing demand for the plant-based drugs in the prevention of diseases has created an urge for the isolation of phytochemicals with amelioration of health issues and enhance the lifestyle among the patients. The study concluded that the ethanolic extract of Simarouba glauca possess notable anti-inflammatory activity from the inhibition of protein denaturation and COX-2 expression inhibition, which can be attributed to the presence of plethora of bioactive compounds present in the plant. The results validated the previous findings and confirmed the anti-inflammatory property expressed by the plant. Further ample research is indispensable to confirm the potential of the extracts for the biological activities and development of therapeutic agents along the conventional anti-inflammatory drugs. Also, the non-toxic effect and safety issues raised with the consumption of molecules isolated from plant is to be determined.

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AUTHOR'S NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. Authors confirmed that the pa-per was free of plagiarism.

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