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Original Article

EVALUATION OF *CARICA PAPAYA* LEAF EXTRACT IN PLATELET PROPAGATION FROM STEM CELLS

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ABSTRACT

Objective: To evaluate the efficiency of *Carica papaya* extract in differentiating stem cells into platelets**.**

Methods: The bioactive compounds of *C. papaya* leaf extract were screened by biochemical and LCMS-MS methods. Mesenchymal stem cells (MSCs) were cultured with and without *C. papaya* leaf extract and observed for megakaryocyte-mediated platelet differentiation. The microscopy and flow cytometer analysis were performed from day 0 to day 12.

Results: The biochemical and LCMS-MS screening of *C. papaya* leaf extract confirmed the presence of alkaloids, saponins, glycosides, steroids, flavonoids, phlobatanins and anthracyanine. When treated with leaf extract (50µg), the MSCs differentiated into megakaryocytes and platelets.

Conclusion: The present study has shown the effect of *C. papaya* leaf extract in MSCs differentiating into platelets. Since the crude extract of the leaf was used, the bioactive compound(s) responsible for platelet production is yet to be confirmed.

Keywords: Platelet production, *Carica papaya* leaf extract, Dengue virus, Alkaloids and flavonoids

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INTRODUCTION

Carica papaya, a herbaceous plant belonging to the *Caricaceae* family, is one of the most popular and economically important plants in the world as a food source and is widely used as a traditional medicinal plant for treating various diseases [1]. Traditionally papaya plants have been used as anthelmintic, digestive disorders, diarrhoea, skin diseases, male contraceptives, and raw materials for cold [2-4]. *Carica papaya* contains the enzyme papain in the fruits, stems and leaves. Phytochemicals in *C. papaya* have been shown to increase the immune system and promote the release of natural chemicals with antitumor activity [5]. The mature leaf concentrate from the Sri Lankan wild-type cultivar of *C. papaya* modulates both the nonfunctional and functional immune responses of Wistar rats [6].

Thrombocytopaenia management during common and potentially life-threatening dengue infection is considered the most important clinical finding on the efficacy of *C. papaya* leaf [7]. The methanol extract of *C. papaya* leaf has been shown to have many phenolic compounds with antioxidant properties [8]. In addition, the alkaloid carpaine was reported to be a significant contributor to anti-thrombocytopaenic properties [9]. It has been demonstrated that the leaf extract of C. papaya augmented platelet count in various dengue fever models in animals, along with reduced clotting time in thrombocytopenic rats [10, 11]. The mature leaf concentrate of *C. papaya* of the red lady cultivar grown in Sri Lanka increased the platelet and total white blood cells (WBC) activity in hydroxyurea-induced thrombocytopenic Wistar rat model as well as in normal counterpart rats [12].

Embryonic stem cells (ES) are pluripotent cells derived from preimplantation embryos. ES cells can be maintained in culture indefinitely as undifferentiated cells, which can form more differentiated cell types and organs [13, 14]. Human ES cells provide a unique, homogenous, unlimited starting population for studying human hematopoiesis. Human ES cells can be cultured for at least 300 populations without observed senescence while maintaining normal karyotypes, telomere lengths and pluripotency. Moreover, these cells can be cloned from a single cell without losing pluripotency [15, 16]. Human ES cells give rise to differentiated cells and tissues or teratomas in immunodeficient mice or embryoid bodies *in vitro* [17]. Mouse and human ES cells differ in morphology, population doubling time and growth factor requirements [18, 19]. Mouse ES cells can be maintained as undifferentiated "feeder-independent" cells if growth factors such as leukaemia inhibitory factor (LIF) or related cytokines are added to the media. When human ES cells are grown without feeder cells and with LIF, they either differentiate or die.

Hematopoiesis is sustained by a pool of hematopoietic stem cells (HSCs) which are extensively self-renewed and differentiated into hematopoietic progenitor cells (HPCs) [20]. HPCs are committed to specific lineage and are functionally defined as colony-forming units (CFUs) or burst-forming units (BFUs), i.e., HPCs of the erythroid series (BFU-E, CFU-E), the megakaryocytic lineage (BFU-MK, CFU-MK), the granulocytes-monocytic series (CFU-GM) [21, 22]. Early HPCs also circulate in peripheral blood. Thrombopoietin (TPO) regulates the production of megakaryocytes when platelets are below the normal level. TPO cytokines bind with the TPO receptor, activate JAK and STAT pathways, and stimulate the production of megakaryocytes [23].

The extract from *C. papaya* leaves increased plasma monocyte chemoattractant protein-1 (MCP-1) levels during the peak of viremia when given orally to AG129 dengue-infected mice. This suggested the possible immunomodulatory capacity of this plant during DENV infection [24].

Clinical studies have shown that *C. papaya* leaf extract increased platelet counts in patients suffering from dengue [25-27]. Papaya leaves contain phenolic compounds, papain and alkaloids, and these nutrients act as potent antioxidants, enhancing the body's immunity. In addition, acetogenin, a compound found in papaya leaves, helps prevent diseases like malaria and dengue. However, dengue fever is one of the major vector-borne diseases, and appropriate prevention and control measures based on natural products have yet to be developed. Therefore, this study evaluated the action of *C. papaya* leaf extract on stem cell differentiation into platelet.

MATERIALS AND METHODS

Materials

The young leaves of *Carica papaya* were collected from the Somwarpet town area in Karnataka, India and washed with distilled

water to remove contamination. Then, they were air-dried, cut into pieces, pulverized into powder and stored in a polyethylene bag. The powder (45g) was extracted with 350 ml of solvent (water/methanol/ethanol) in a soxhlet apparatus. The final concentrate was evaporated, and 2.5g of final powder was obtained. 2.5 mg was dissolved in 2.5 ml water (1 mg/ml) and used for analysis.

The chemicals acetic acid, chloroform, ferric chloride ($FeCl_3$), sulfuric acid (H_2SO_4), hydrochloric acid (HCl) were purchased from Qualigens Fine Chemicals, India.

Phytochemical qualitative analysis

The phytochemical analysis was performed by standard methods [28-31].

Test for saponins: Distilled water was mixed and added to an aqueous extract (1 mg/ml) and mixed vigorously. The frothing obtained was mixed with a few drops of olive oil and mixed vigorously. The appearance of the foam showed the presence of saponins.

Tests for glycosides

Liebermann's test: Acetic acid and chloroform (1:1 v/v) was added to aqueous extract (1 mg/ml). This mixture was then cooled and added with concentrated H_2SO_4. The green colour represented the entity of aglycone, a steroidal part of glycosides.

Keller-kiliani test: Glacial acetic acid and 2.0% $FeCl_3$ mixture (4:1) was mixed with the aqueous plant extract (1 mg/ml) and conc. H_2SO_4. Cardiac steroidal glycosides exhibited a brown ring formed between the layers.

Salkowski's test: Conc. H_2SO_4 was added to an aqueous plant extract (1 mg/ml). A reddish brown colour formed, which indicated the presence of steroidal aglycone part of the glycoside.

Test for steroids: Chloroform and conc. H_2SO_4 (20:1) was added to aqueous plant extract (1 mg/ml). In the lower chloroform layer, the red colour appeared, indicating the presence of steroids.

Test for tannins: 5% ferric chloride was added to aqueous plant extract (1 mg/ml). The formation of dark blue or greenish black indicates the presence of tannins.

Test for Alkaloids

Wagner's test: Diluted HCl and Wagner's reagent were added to aqueous plant extract (1 mg/ml) and shaken well.

Test for flavonoids: 2N sodium hydroxide was added to aqueous plant extract (1 mg/ml). The presence of yellow colour indicates the presence of flavonoids.

Test for quinones: Conc. H_2SO_4 was added to an aqueous plant extract (1 mg/ml). The red colour indicates the presence of quinones.

Test for phenols: Distilled water and a few drops of 10% $FeCl_3$ were added to aqueous plant extract (1 mg/ml). The formation of blue or green colours indicates the presence of phenols.

Test for terpenoids: Aqueous plant extract (1 mg/ml) was treated with chloroform and conc. H_2SO_4. The formation of red-brown colour at the interface indicates the presence of terpenoids.

Test for coumarins: 10% sodium hydroxide was added to aqueous plant extract (1 mg/ml). The appearance of yellow colour indicates the presence of coumarins.

Test for anthraquinones: 10% ammonia solution was added to aqueous extract (1 mg/ml), and the appearance of a pink precipitate indicates the presence of anthraquinones.

Test for phlobatannins: 2% hydrochloric acid was added to the aqueous extract (1 mg/ml). The appearance of a red colour precipitate indicates the presence of phlobatannins.

Test for anthracyanine: 2N sodium hydroxide was added to aqueous extract (1 mg/ml) and heated for 5 min at 100 °C. The formation of bluish-green colour indicates the presence of anthocyanin.

LCMS-MS analysis of papaya leaf extract

The *C. papaya* leaf extracts (1 mg/ml) (aqueous, ethanol and methanol) were subjected to LCMS-MS (Shimadzu 8050) analysis. The parameters were: Esi mode, solvent methanol, mobile phase A-5 mm ammonium formate, Mobile phase B-Methanol, injection volume 10 µl. In the graph obtained, individual peaks were compared with available literature for compound identification. The compounds thus identified were compared and tabulated.

Preparation of aqueous extract of *C. papaya* leaf for cell culture

Middle-aged, fresh *C. papaya* leaves were collected, washed and dried under shade. Of the dried leaves, the middle stems were removed, and the rest were crushed using a mortal and pestle. The crushed powder of 45 grams was added to 350 ml of distilled water and kept for 16 h at 60 °C. This extract was cooled, filtered and used for cell culture experiments.

Culture of mesenchymal stem cells

Vented tissue culture flasks (25 cm^2) with 10 ml Dulbecco's modified Eagles medium (DMEM) with 10% fetal calf serum (FCS) were seeded with $1X10^7$ cells for primary culture. The flasks were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and were given by half MSC medium change every week until the fibroblast-like cells at the base of the flask reached confluence.

Culture of mesenchymal stem cells by addition of *C. papaya* leaf extract

Bone Marrow Stem cells were cultured in DMEM supplemented with Fetal Bovine Serum (10%) and 50 µl of *C. papaya* leaf extract (1 mg/ml) in the presence of antibiotics (Penicillin-Streptomycin, 1%). Cells were incubated at 37 °C in the presence of 5% CO_2. This culture with *C. papaya* leaf extracts was carried out in quadruple. Cultures without *C. papaya* leaf extract were considered as controls. From day 0, the cells were observed under the fluorescence microscope, and their morphology and cell count results were documented.

Flow cytometer analysis of platelets derived from MSC cells

The number of platelets produced through MSCs-derived megakaryocytes were determined by flow cytometer using MWReg 30 monoclonal antibody1C2, a platelet-specific antibody. The culture medium was gently collected and centrifuged at 150*g* for 20 min to remove the large nucleated cells. The supernatant was fixed with 1% paraformaldehyde for 1 hour and centrifuged at 900*g* for 10 min. Next, the cell pellet was washed with Hanks balanced salt solution with Ca^{2+}(HBSS) containing 1% FBS and incubated with 10g/ml MWReg 30 monoclonal antibody1C2 (Seikagaku, Tokyo, Japan), followed by FITC–goat anti-rat IgG; each incubation was performed on ice for 1 hour. Finally, the cells were washed and analyzed by a flow cytometer. A single platelet gate was created by analyzing adult mouse peripheral platelets similarly.

RESULTS

The qualitative tests were conducted to evaluate the phytochemical profile (alkaloid, tannin, flavonoid, saponins, tannins and Glycoside) of *C. papaya* leaf extract. The results are presented in table 1. The phytochemical screening of aqueous, ethanolic and methanolic extracts showed the presence of saponins, glycosides, steroids, tannins, flavonoids, quinines, terpenoids, coumarins, phlobatanins and anthraquinones. However, according to the earlier report, phenol was detected only with methanolic extracts [32].

The LCMS-MS analysis of the phytochemicals present in *C. papaya* leaf extract with their corresponding retention time, molecular formula, molecular weight, and relative abundance are presented in fig. 1 and table 2. The aqueous extract was found to have more compounds, followed by the methanolic extract. Few compounds like flavinoids, alkaloids and quinines are absent in ethanolic extract. Anthracyanine was present in both aqueous and ethanolic extracts.

Table 1: Phytochemical qualitative analysis of *Carica papaya* leaf extract (1 mg/ml)

S. No.	Tests	Aqueous	Ethanolic	Methanolic
1	Saponins	+	+	+
2	Glycosides			
	Liebermann's Test	-	+	+
	Keller-Kiliani Test	-	+	-
	Salkowski's Test	+	-	-
3	Steroids	+	+	+
4	Tannins	+	+	+
5	Alkaloids			
6	Flavonoids	+	+	-
7	Quinones	+	+	+
8	Phenols	-	-	+
9	Terpenoids	+	-	+
10	Cardiac Glycosides	+	+	+
11	Coumarins	+	+	+
12	Anthraquinones	-	-	-
13	Phlobatannins	+	-	+
14	Anthracyanine	+	-	+

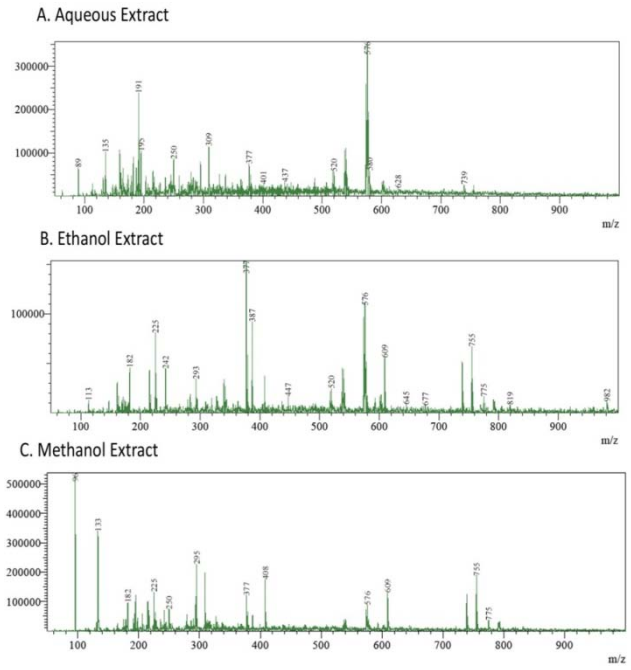

Fig. 1: LC-MS chromatogram analysis of A. aqueous, B. ethanol and C. methanol extract of *Carica papaya* leaf

Table 2: LCMS-MS analysis of aqueous ethanol and methanol extract from *Carica papaya* leaf

S. No.	Tests	m/Z (Mass to charge)	A (Water) intensity		B (Ethanol) intensity		C (Methanol) intensity	
			Absolute	Relative	Absolute	Relative	Absolute	Relative
1	Saponins	789 (mol. wt-1223.3g/mol)						
2	Glycosides (aglycone)	389 (mol wt-526.5 g/mol)	16297	5.00	8343	5.53		
3	Steroids	425 (mol. wt-526.5 g/mol)	16543	7.84	8579= (421.3 m/Z)	5.69= (421.3 m/Z)	492827= (429.45 m/Z)	8.74= (429.45 m/Z)
4	Tannins	371 (mol. wt-636.5 g/mol)	19897= (373.30 m/Z)	6.11= (373.30 m/Z)				
5	Alkaloids	319 (mol. wt-g/mol)	17381	5.34			37367= (313.10 m/Z)	7.06 =(313.10 m/Z)
6	Flavonoids	151 (mol. wt-222.24g/mol)	21704	6.08				
7	Quinones	189 (mol.-108.09 g/mol)	27956	7.84			41683 =(191 m/Z)	7.87 =(191 m/Z)
8	Phenols	135 (mol. wt-94.11g/mol)	101715	28.51			338784= (133.10 m/Z)	63.97 = (133.10 m/Z)
9	Terpenoids	136 (mol. wt-552.8g/mol)	92418	28.37	559251= (138.10 m/Z)	18.12= (138.10 m/Z)	1193351 = (138.10 m/Z)	21.16= (138.10 m/Z)
10	Cardiac glycosides	591 (mol. wt-780.9g/mol)						
11	Coumarins	147 (mol. wt-146.14 g/mol)	26278	7.37	12345	8.18		
12	Anthraquinones	270 (mol. wt-208.21 g/mol)	14583= (271 m/Z)	6.92= (271 m/Z)	18532= (266.15 m/Z)	6.00	266.15 185328 6.00	266.15 185328 6.00
13	Phlobatannins	278.118 (mol. wt-g/mol)	19292	9.15	3087055 =(279.20 m/Z)	100.00 =(279.20 m/Z)	54520	10.29
14	Anthracyanine	449 (mol. wt-207.24g/mol)	10673	5.06	16983 =(447.30 m/Z)	11.26 =(447.30 m/Z)		

Bone marrow mesenchymal stem cells (MSCs) cultured in DMEM were subjected to platelet differentiation with and without *C. papaya* leaf extract (fig. 2). In the absence of the extract, the stem cells proliferated into dense colonies and formed a confluence layer without any differentiation during the 12 d study period. However, while exposed to *C. papaya* leaf extract, the stem cells started differentiating to larger colonies on day 6, progressed to megakaryocytes on day 9 and matured into platelets on day 12.

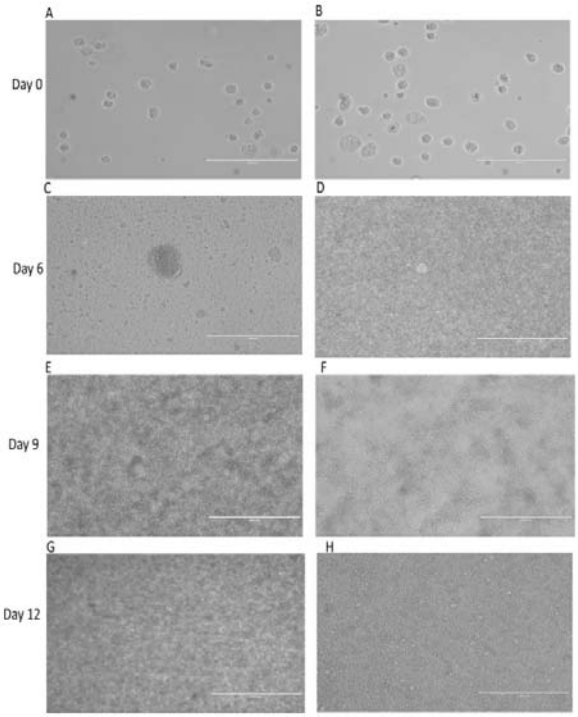

Fig. 2: Effect of *Carica papaya* leaf extract in Mesenchymal Stem Cells culture. Mesenchymal Stem Cells were cultured in the presence of *C. papaya* leaf extract (1 mg/ml) and evaluated against culture without extract. A: Control-MSCs proliferated without any differentiation. B: *C. papaya* leaf extract triggered the differentiation of MSCs into larger cells. C: MSCs formed uniform monolayer without *C. papaya* leaf extract. D: The differentiation of MSCs further progressed into larger colonies. E and G: MSCs proliferated into dense colonies and formed a confluence layer. F: The larger colonies progressed into megakaryocytes. H: Maturation of megakaryocytes into platelets

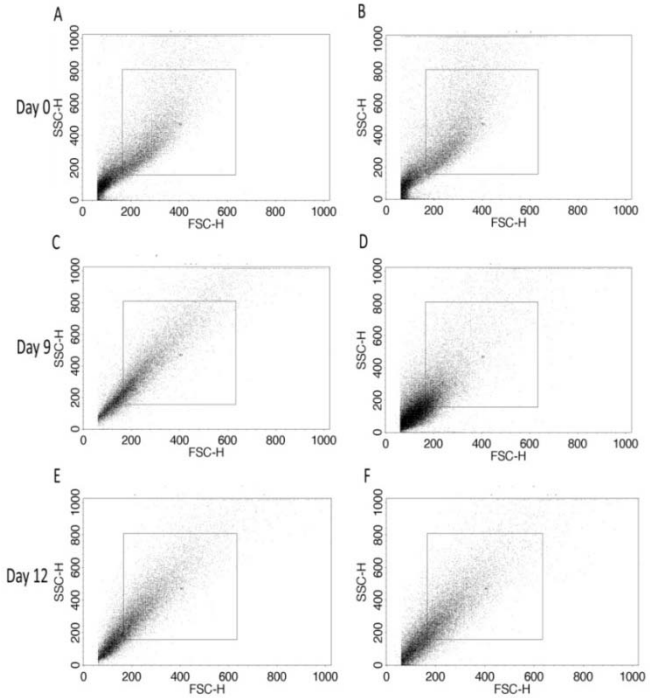

Fig. 3: Flow cytometer analysis of mesenchymal stem cells culture with *C. papaya* leaf extract. Mesenchymal Stem Cells were cultured in the presence of *C. papaya* leaf extract (1 mg/ml) and evaluated against culture without the extract. Day 0: A-Control MSCs. B: MSCs exposed to *C. papaya* leaf extract. Day 9: C-MSCs formed uniform proliferation without *C. papaya* leaf extract. D: The different MSCs into larger colonies. Day 12:. E-MSCs formed a confluence layer without differentiations. F-Maturated platelets from megakaryocytes

The MSCs treated with *C. papaya* leaf extract were analyzed by flow cytometer using MWReg 30 monoclonal antibody1C2, a platelet-specific antibody (fig. 3). This further confirmed the differentiation of the MSCs to megakaryocytes and platelets (D and F, respectively) by the extract. However, no differentiation was observed with the MSCs without exposure to *C. papaya* leaf extract (C and E, respectively).

DISCUSSION

Thrombocytopenia, a critically reduced platelet count, is associated with severe dengue fever, thus, a significant mortality factor [33]. The viral pathogen replicates in platelet, declines platelet production, and destroys the platelets in circulation. Several reports based on the *in vitro* studies have affirmed an increase in the platelet number with the administration of *C. papaya* leaf extract [34]. The leaves of *C. papaya* have been found to have multiple chemical compounds, namely alkaloids, terpenoids, phenols, tannins, flavonoids, saponins and glycosides [7, 9, 35]. Among the phytocompound (s), the alkaloids, particularly the "carpaine" alkaloid, rather than phenolic compounds, are responsible for the anti-thrombocytopenic activity. The present study also identified the compounds as per the earlier reports. As expected, flavonoids and other alkaloids were found to be more and higher in concentration, and the phenol with less amount in aqueous and absent in ethanolic extract.

Treating dengue patients with *C. papaya* leaf extract has been shown to potentially inhibit intracellular replication of DENV-2 with a significant reduction ($p<0.05$) in platelet aggregation [26] and also the reversal of peripheral platelet destruction by membrane stabilization [36]. *C. papaya* leaf flavonoids have been shown to inhibit a protease involved in viral assembly [25]. The saponins were considered to enhance cell-mediated immunity and humoral antibody in animal models [37]. The antioxidants with free radical scavenging properties of papaya leaf extract may significantly prevent hemolysis and bleeding [38]. Most of these studies were on platelet protection or increased production, while studies on stem cell differentiation into platelet are scanty. The present study evaluated the effect of *C. papaya* leaf extract in bone marrow-derived mesenchymal stem cells differentiation into platelet. The results have delineated that platelets can be generated through the promegakaryocyte pathway by treating the stem cells with *C. papaya* leaf extract. An earlier report showed that *C. papaya* leaf extract increased the expression ALOX 12 gene by 15-fold and platelet-activating factor receptor (PTAFR) genes. The expression of these genes increased megakaryocyte production and its conversion into platelets. Activation of the 12-HETE mediated pathway could be the mechanism of action in the production of platelets [39, 40]. It is identified that about 60% of the anticancer compounds occurred in anticancer drugs are derived from herbal sources [41].

CONCLUSION

This study is a preliminary milestone in platelet production by differentiating the stem cells with *C. papaya* leaf extract. There is a volume of bioactive compounds in *C. papaya* leaf extract. As supported by earlier studies, many play an essential role in platelet production, protection, and viral pathogen destruction. However, specific compound(s) and their mechanism have not been well established. While most studies were on platelet regeneration and protection under *in vivo* conditions, stem cell-mediated platelet production has yet to be well studied. We initiated the MSCs differentiation into megakaryocyte-mediated platelets with *C. papaya* leaf extract in this study. Our study has provided valuable information on the positive effects that stem cells can be used for large-scale platelet production, thus reducing the burden on whole blood-based platelet collection. Further studies are required to delineate the specific compounds and their action on stem cell differentiation into platelets.

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AUTHORS CONTRIBUTION

This is author's sole research work and each author has contributed equally. This research work does not have contribution from others.

CONFLICT OF INTERESTS

Declared none

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