Research Article

# Sappan Wood Ethanol Extract (*Caesalpinia sappan* L.) as Adjuvant and Substitute of Iron Chelator in Acute Iron Overload Rat Model

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**Abstract** | Excessive iron accumulation in the body causes oxidative stress, which can lead to cell death. Iron chelating agents such as deferoxamine, deferiprone, and deferasirox have been used to treat iron overload, but their uses are inconvenient caused by their side effects. Therefore, the invention of an alternative chelating agent with a more efficient effect, convenient uses, and affordable is essential, which can be done with natural plants. Sappan wood (*Caesalpinia sappan* L.) contains active compounds, such as brazilin and flavonoid with the ability to chelate Fe. This study aims to determine the effect of sappan wood ethanol extract (SWEE) as an adjuvant and substitute for iron chelator. The adjuvant test group was given 60 mg/kg BW iron dextran (ID), 75 mg/kg BW deferiprone (DFP), and 50 to 200 mg/kg BW SWEE, while the substitute group was administered with 60 mg/kg BW ID and DFP:SWEE with a gradient ratio. The ID was given orally for the first 14 days at intervals of 3 days, while DFP and SWEE were administered orally every day for 28 days. The parameters observed include iron levels in the serum, liver, heart, spleen, kidney, and brain. The results showed that the effective dose was the combination of 75 mg/kg BW DFP and 50 mg/kg BW SWEE as an adjuvant, while 200mg/kg BW SWEE can be used as a substitute for chelators, especially in the heart. The decrease in serum and organ iron levels due to the combination of DFP and SWEE showed their ability to act as an adjuvant and substitute for chelators.

**Keywords** | Adjuvant, Deferiprone, Iron levels, Sappan wood ethanol extract, Substitute.

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## INTRODUCTION

Iron overload is a phenomenon caused by the accumulation of excess iron (Fe) in the body. Regular blood transfusion therapy in thalassemia patients can induce its accumulation (Mishra and Tiwari, 2013). Furthermore, the inability to produce hepcidin in hereditary hemochromatosis also causes this condition due to excess absorption (Kowdley et al., 2019). Iron can act as an electron carrier and catalyst for different processes, such as the Fenton reaction (Gattermann et al., 2021). The reaction often leads to the production of hydroxyl free radicals known as reactive oxygen species (ROS) that can induce oxidative stress and lipid peroxidation when their level exceeds that of antioxidants (Sousa et al., 2020). Iron overload in the body causes damage to body organs, such as the liver, heart, spleen, kidney, and brain (Kolnagou et al., 2013; Karunaratna et al., 2017; Hashemieh, 2019; Reinert et al., 2019). Removal of

excess Fe can be carried out with the use of chelators, such as deferoxamine, deferiprone, and deferasirox (Syobri et al., 2020). Deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one) is an oral bidentate chelator that chelates iron in a ratio of 3:1 (ligand:iron). The deferiprone-iron complex is then excreted in urine (Hider and Hoffbrand, 2018), but it has several side effects, such as gastric intolerance, agranulocytosis, and neutropenia (Kontoghiorghes et al., 2020). Therefore, the discovery of an alternative chelating agent that is more efficient, convenient, and affordable is essential, such as the use of natural plants.

Sappan wood (*Caesalpinia sappan* L.) is a woody shrub, which belongs to the Leguminosae family. It is often used as herbal medicine to treat diarrhea, tumors, syphilis, and malaria (Sari and Suhartati, 2016). The wood also has several bioactivities, such as antioxidant, anti-inflammatory, antibacterial, and vasorelaxant activities (Nirmal et al., 2015). Furthermore, it can exert cardioprotective effects due to its properties and can potentially be used to treat cardiovascular disorders (Syamsunarno et al., 2021). Several studies have also explored its active components including alkaloids, flavonoids, brazilin, saponins, monoterpenes, and diterpenes. The active substances that act as antioxidants are flavonoids and brazilin (Nirmal et al., 2015). Brazilin can bind to different transition metals, such as Fe, Mg, Cu, and Zn (Zulenda et al., 2019). Sappan wood ethanol extract (SWEE) acts as a chelator that decreases ferritin, as well as iron levels in the serum and liver. It can also increase transferrin in iron overload model rats (Safitri et al., 2018). Adjuvant therapy is a treatment that has been used to induce the effectiveness of the main medication when used together. In adjuvant therapy, additional drugs are given after the main drug seems to be less effective so that the effect of the treatment can be better. Substitution therapy is a treatment that has been used to replace the main medication, in which the substitute drugs have the same ability as the main drug either it has the same formulation as the main drug or it has the same molecule ability (Johnston et al., 2011). Previous studies have explored SWEE potential as an iron chelator in vivo, but further optimization to determine the dose at which SWEE as a substitute iron chelator or the combination of SWEE + deferiprone as an adjuvant standard regiment can effectively prevent organ damage caused by iron accumulation has not been carried out. There is also no study on its use as an adjuvant and substitute for chelators. Therefore, this study aims to determine the effect and optimum dose of SWEE as an adjuvant and iron chelator substitute.

## MATERIALS AND METHODS

### Sappan Wood Extraction

Sappan wood was obtained from the Yogyakarta Forestry and Plantation Service, Yogyakarta Special Region. Furthermore, the heartwood of sappan wood. was processed into powder form simplicia and macerated using 96% ethanol for 4 x 24 hours. The liquid extract was then evaporated with a rotary evaporator at 60°C to obtain a dry extract. It was dissolved in distilled water to obtain doses of 50, 100, 150, and 200 mg/kg BW.

### Animal Treatment

This study was approved and registered with the Research Ethics Commission of Padjadjaran University with Ethical Clearance number 605/UN6.KEP/EC/2021. Seven weeks old healthy male Wistar rats (*Rattus norvegicus*) with an average body weight of 200-300 grams were used for the experiment. The test animals were acclimatized for 7 days at room temperature and irradiation time of 12 hours light and dark (OECD, 2008). Foods and drinks were administered *ad libitum*, and they were then randomly divided into eleven groups. The normal, negative, and positive controls were given aquadest, 60mg/kg BW iron dextran (ID) (Hemadex, Sanbe, Bandung, Indonesia), and 60mg/kg BW ID + 75mg/kg BW deferiprone (DFP), respectively. The adjuvant group consists of four test subgroups, which were administered with 60mg/kg BW ID and 75mg/kg BW DFP along with different concentrations of SWEE, namely 50 mg/kg BW (A1), 100 mg/kg BW (A2), 150 mg/kg BW (A3), and 200 mg/kg BW (A4). The substitute test group consisted of four subgroups, which were given 60mg/kg BW ID and dosage variations of 75% DFP and 25% SWEE (S1), 50% DFP and 50% SWEE (S2), 25% DFP and 75% SWEE (S3), as well as 100% SWEE (S4). The oral gavage administration of 60 mg/kg BW as accumulative dose iron dextran was carried out for 14 days with an interval of once every 3 days, while deferiprone and SWEE were administered every day for 28 days, orally.

### Samples Collection

On day 28, the rats were fasted for 24 hours and then sacrificed with ketamine-xylazine at dose of 0.2 ml/100 grams BW intramuscularly on day 29. Blood samples were taken intracardially using a 3cc syringe and placed in a venoject tube for serum production. The rat's liver, heart, spleen, kidney, and brain were separated and washed using 0.9% NaCl.

### Serum Iron Levels Assay

The blood samples were centrifuged at 3000 rpm for 10 minutes to obtain serum, which was used for the serum iron levels assay. Measurement of Fe concentration was carried out using colorimetry method with ferene. Iron Fs Ferene reagent (Catalog No. 1 1911 99 10 021) was obtained from DiaSys (Holzheim, Germany). The process was performed based on the procedure in the manufacturer's working protocol.

### Organ Iron Levels

The measurement of organ iron levels was carried out using the Atomic Absorption Spectrophotometry (AAS) method. A total of 0.2 g of the samples was weighed, placed in a beaker glass, and 5ml of 65% $HNO_3$ was added. The organs were heated until they were dissolved, followed by the addition of 2 ml of 30% $H_2O_2$. Subsequently, the mixture was heated again until gas bubbles form were seen. The solution was diluted using aquadest to obtain a total volume of 25 ml in the volumetric flask. It was then homogenized and measured using AAS (AAnalyst 400, USA) with a wavelength of 248.3 nm.

### Statistical Analysis

Statistical analyzes were carried out using the Statistical Package for the Social Sciences version 26.0 software (IBM Corp., Armonk, NY, United States) and the graph was visualized with GraphPad Prism version 8.0.1. software. The data were tested with the normality test, followed by one-way analysis of variance (ANOVA) and Duncan post hoc test to evaluate significant differences when they are normal. Meanwhile, when the data are not normal, the Kruskal-Wallis and Mann-Whitney tests were performed. The analysis was carried out at a 95% confidence level ($p < 0.05$).

## RESULT

### Serum Iron Levels

The increase in serum iron levels of the negative control (194.31 mg/dL) was 63% higher than the normal control (118.93 mg/dL), as shown in Figures 1A and 1B. Furthermore, the levels in the adjuvant group were not significantly different, but there was a decrease in serum iron compared to the negative and positive controls (174.97 mg/dL), especially in the A3 (141.14 mg/dL) group, which was 27% lower than the negative control, as shown in Figure 1A. The iron concentration in the S1 (149.41 mg/dL), S3 (143.44 mg/dL), and S4 (140.49 mg/dL) groups were significantly lower than the negative control. S1 was 23% lower than the negative control and can potentially be used as an effective dose.

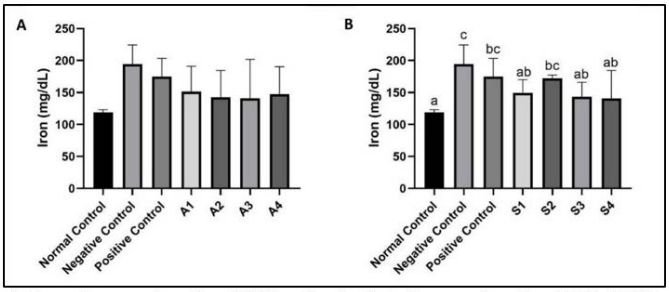


**Figure 1:** Adjuvant serum iron level (A) and substituted serum iron level (B). Different letters between groups showed significant differences with 95% confidence level ($p < 0.05$).

### Liver Iron Concentration

Increased liver iron concentration was observed in the negative control (236.25 ppm), which was 40% higher than the normal control (161.74 ppm), as shown in Figures 2A and 2B. The combination of SWEE and deferiprone in the adjuvant test group significantly reduced Fe levels compared to the negative control, as shown in Figure 2A. Furthermore, A1 (153.49 ppm) had the lowest liver iron, which was 35% lower than the negative control. The combination of SWEE and deferiprone in the substitute test group significantly reduced liver iron concentration, especially in the S3 (114.57 ppm) group, which was 52% lower than the negative control, as shown in Figure 2B.

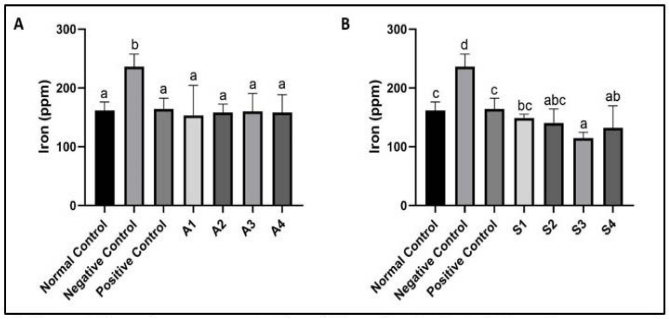


**Figure 2:** Adjuvant liver iron concentration (A) and substituted liver iron concentration (B). Different letters between groups showed significant differences with 95% confidence level ($p < 0.05$).

### Cardiac Iron Concentration

Increased cardiac iron concentration was observed in the negative control (307.86 ppm), which was 199% higher than the normal control (102.9 ppm), as shown in Figures 3A and 3B. The combination of SWEE and deferiprone in the adjuvant test group reduced the concentration compared to the negative control with an effective dose in the A1 (107.11 ppm) group, which was 65% lower than the negative control, as shown in Figure 3A. A1 also has the same ability as A4 (100.57 ppm), which had the lowest value. The combination of SWEE and deferiprone in the substitute test group showed a significant decreasing trend compared to the negative control, as shown in Figure 3B. The S4 (104.14 ppm) group was 66% lower than the negative control.

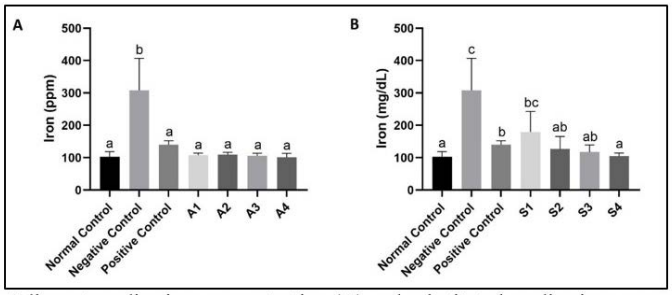


**Figure 3:** Adjuvant cardiac iron concentration (A) and substituted cardiac iron concentration (B). Different letters between groups showed significant differences with 95% confidence level ($p < 0.05$).

### Spleen Iron Concentration

Increased levels of spleen iron concentration were observed in the negative control (446.99 ppm), which was 28% higher than the normal control (350.05 ppm), as shown in Figures 4A and 4B. The concentration in the adjuvant test group did not show a significant difference, as shown in Figure 4A. However, there was a decrease in spleen iron in A2 (360.01 ppm), which was 19% lower than the negative control and can potentially be used as an effective dose. The combination of SWEE and deferiprone in the substitute test group also did not show a significant difference from the negative control, as shown in Figure 4B. The iron level in the S2 (406.05 ppm) group was 9% lower than the negative control due to the administration of SWEE and deferiprone.

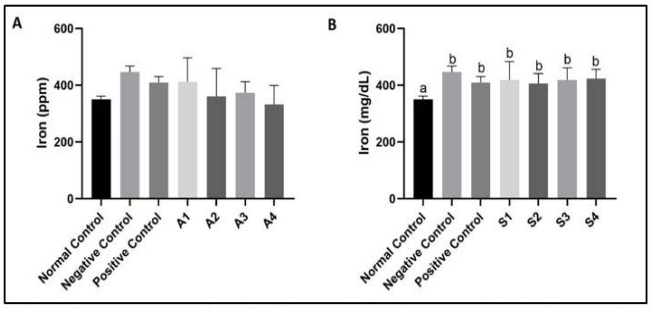


**Figure 4:** Adjuvant splenic iron concentration (A) and substituted spleen iron concentration (B). Different letters between groups showed significant differences with 95% confidence level ($p < 0.05$).

### Kidney Iron Concentration

Increased renal iron concentration was observed in the negative control (280.86 ppm), which was 219% higher than the normal control (88.11 ppm), as shown in Figures 5A and 5B. The combination of the SWEE and deferiprone in the adjuvant test group showed a significant decrease compared to the negative control, as shown in Figure 5A. There was a 50% decrease in kidney iron level in the A1 (140.01 ppm) and it can potentially be used as an effective dose. The combination of SWEE and deferiprone in the substitute test group showed a significant increase in renal iron levels in S1 (437.65 ppm), S2 (461.1 ppm), and S3 (455.61 ppm) compared to the negative controls, as shown in Figure 5B.

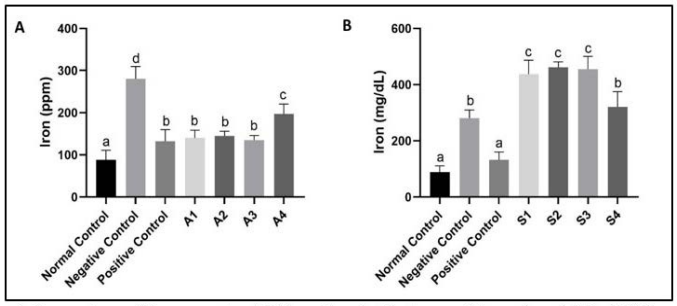


**Figure 5:** Adjuvant renal iron concentration (A) and substitute renal iron concentration (B). Different letters between groups showed significant differences with 95% confidence level ($p < 0.05$).

### Brain Iron Concentration

Increased brain iron concentration was observed in the negative control (66.63 ppm), which was 22% higher than the normal control (54.54 ppm), as shown in Figures 6A and 6B. The levels in the adjuvant and substitute groups were not statistically significant, as shown in Figures 6A and 6B, respectively. However, there was a decrease in brain iron after the administration of SWEE and deferiprone in both groups. The concentration in A1 (54.04 ppm) was 19% lower than the negative control, while the S1 (35.31 ppm) group was 47% lower.

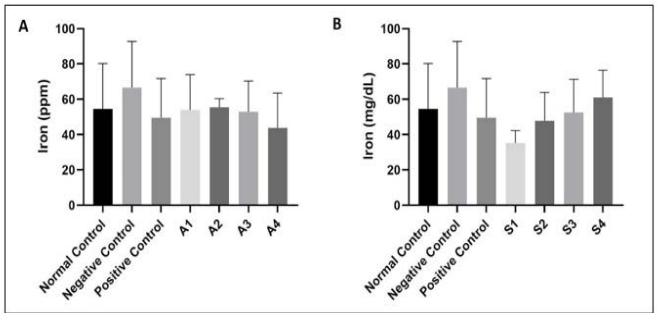


**Figure 6:** Adjuvant brain iron concentration (A) and substitute brain iron concentration (B). Duncan post-hoc test were not performed ($p > 0.05$).

## DISCUSSION

Maskoen et al. (2016) reported that the administration of 60mg/kg BW iron dextran orally increased iron levels, which led to iron overload in rats. This condition induced the Fenton reaction, which is a reaction between $H_2O_2$ and $Fe^{2+}$ to produce hydroxyl free radicals known as ROS. Furthermore, ROS can induce oxidative stress and lipid peroxidation when their concentration exceeds that of antioxidants (Sousa et al., 2020). Iron overload causes an increase

in serum iron levels (Peng and Uprichard, 2017). This was illustrated in Figure 1A and 2B, where there was increased serum levels in the negative control. A decrease in serum iron occurred due to the administration of deferiprone and SWEE, especially in A3 and S1. Excess Fe can accumulate in body organs, such as the liver, heart, spleen, kidney, and brain.

The liver plays an important role in iron metabolism, and it consists of 80% hepatocytes, which serve as the main storage site in the body. Hepatocytes can store iron up to 20-30% of the total body iron (Perdana and Jacobus, 2015). Furthermore, non transferrin-bound iron (NTBI) is one of the major contributors to deposition in these cells (Vogt et al., 2021). Based on the results, there was an increase in the iron content of the negative control. A decrease in concentration occurred due to the combination of deferiprone and SWEE, especially in A1 and S3 treatment groups. The administration of deferiprone can eliminate iron from the liver in overload condition (Kontoghiorghe and Kontoghiorghes, 2016).

The heart is one of the main organs affected by this condition (Taher and Saliba, 2017) because cardiac myocytes are the main consumers of oxygen. In cardiac oxygen metabolism, iron mediates the oxidation-reduction reactions. Iron also has a role in electron transfer and oxygen activation during oxidative phosphorylation. Labile iron pool (LIP) is also required in oxygen activation by oxygenation and redox signaling catalysts. Consequently, intracellular balance is required to maintain the aerobic activity of the heart. Reactive oxygen species are continuously produced, which leads to organ damage due to oxidative stress (Lakhal-Littleton, 2019; Mancardi et al., 2021). The results showed that there was a significant increase in the cardiac concentration of the negative control, but a significant decrease occurred in the deferiprone and SWEE treatment, especially in A1 and S4. Deferiprone can serve as a good chelator for cardiac iron concentration (Kontoghiorghe and Kontoghiorghes, 2016; Aggarwal and Mirgh, 2018), as indicated by this study's result.

The spleen plays an important role in the iron recycling process, and it is the main organ that destroys damaged or old red blood cells through erythrophagocytosis (Vogt et al., 2021). High levels of iron in the spleen can be caused by erythrocyte recycling. However, a non-significant decrease was observed after treatment with deferiprone, SWEE, or their combination, especially in the A2 and S2 treatment groups.

The kidney has high metabolic activities and receives 20-25% output from the heart. It also contains many mitochondria and organelles that are rich in heme iron and iron-sulfur proteins. In the kidney, Fe is filtered by the glomerulus and reabsorbed by the renal tubules. Iron absorbed by the kidney can be in the form of transferrin-bound iron (TBI) and NTBI (Scindia et al., 2019). The increase in kidney concentration was caused by the administration of ID. There was a decrease in the concentration of the adjuvant group, while an increase occurred in the substitute group after the administration of deferiprone and SWEE. Yatmark et al. (2016) revealed that deferiprone can reduce iron in the kidney. However, the decrease, which occurred was not significant. In this study, the renal iron levels in the adjuvant test group decreased after administration of deferiprone and SWEE combination. Their administration in the substitute test group led to an increase in renal concentration. This was caused by the function of the kidney itself, namely the processing of urine before excretion. The iron-deferiprone bound is excreted from the body through the urine. Iron filtered by the glomerulus can be reabsorbed by the tubular system (Yatmark et al., 2016; Kontoghiorghes et al., 2020; Morales et al., 2022).

The availability of Fe is needed regularly by the brain. It also acts as a neurotransmitter, as well as a cofactor for enzymes in different processes, such as Adenosine Triphosphate production, DNA, RNA, protein synthesis, and myelination. Iron overload in the brain can cause oxidative stress, which damages the inner membrane of mitochondria, glial cells, and neurons in the organ, thereby increasing the risk of neurodegenerative disorders (Garton et al., 2016; Reinert et al., 2019). In this study, the brain iron concentration was not significant. However, a non-significant decrease occurred after the administration of both deferiprone and SWEE, especially in A1 and S1. Brazilin has the potential to treat brain tumors due to its anti-inflammatory effect as well as the ability to inhibit cell proliferation and induce apoptosis in glioblastoma (Nirmal et al., 2015). Sappan wood ethanol extract also has neuroprotective activities that can prevent neuronal damage, promote synaptic events, and suppress the activation of neutrophils, microglia, and astrocytes (Wan et al., 2019).

Deferiprone is a bidentate oral chelator that forms an iron complex, which contains three molecules of deferiprone and one molecule of iron. Excretion of excess Fe bound by this compound is carried out by urine (Morales et al., 2022). Its small molecular shape has a neutral charge and hydrophilicity, which makes it easy to penetrate and mobilize excess iron from the tissues of all organs, including the heart and brain (Kontoghiorghes et al., 2020). Based on the results, there was a decrease in iron content after the administration of SWEE and deferiprone as an adjuvant and substitute for iron chelator due to their active compound content and synergy.

The antioxidant ability of sappan wood is supported mainly by its flavonoid and brazilin content. Furthermore, the antioxidative activity of flavonoids is obtained from their molecular structure, which has a phenolic hydroxy group. In capturing free radicals, it can donate hydrogen atoms, which makes the radicals inactive (Arifin and Ibrahim, 2018). The iron-chelating activity is possessed by brazilin, which is the main constituent of sappan wood. This property is caused by its phenolic structure, which can reduce or donate hydrogen atoms or electrons (Nirmal et al., 2015). Brazilin can also to bind transition metals, such as Fe, Mg, Cu, and Zn (Zulenda et al., 2019). The decrease in iron levels in this study is consistent with Safitri et al. (2018), where the administration of SWEE reduced liver and serum levels. Sappan wood ethanol extract can also reduce ferritin levels and transferrin saturation as well as increase TIBC and transferrin concentration in iron-excessed rats. Sappan wood ethanol extract has the potential to be used as an adjuvant and substitute for iron chelator. In this study, the combination of 75mg/kg BW DFP and 50mg/kg BW SWEE 50 mg/kg BW is the best adjuvant dose, while 200mg/kg BW SWEE can be used as a substitute, especially in the heart.

## CONCLUSION

The combination of deferiprone and SWEE worked synergistically in lowering serum and organ Fe levels, especially in the liver and heart of iron overload rats. This shows their ability to act as an adjuvant and substitute for iron chelator.

## ACKNOWLEDGMENT

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this study.

## NOVELTY STATEMENT

This study determines the effect and optimum dose of sappan wood ethanol extract as an adjuvant chelator to deferiprone and as an iron chelators substitute as seen in body organs and serum. To the best of our knowledge, the study of sappan wood ethanol extract as an adjuvant and substitute chelator has never been done before.

## AUTHORS CONTRIBUTION

All authors contributed equally to the manuscript.

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