IMMUNOSTIMULATORY ACTIVITY OF AQUEOUS LEAVES EXTRACT OF CASSIA OCCIDENTALIS ON INNATE AND ADAPTIVE IMMUNE RESPONSE IN MICE


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ABSTRACT

Different types of plants have been studied for their roles in immunostimulation and treatment of other diseases. The study aimed at evaluating the stimulatory activity of aqueous leaf extract of Cassia occidentalis on innate and adaptive immune responses in mice. Study mice were grouped into five and studied for 21 days; group I (control group) received normal saline. While the treatment groups: group II - V received aqueous leaf extract of C. occidentalis at different concentrations (250, 500, 1000, and 1500) mg/kg body weight (BW). The mice were sensitized and challenged with sheep red blood cells (SRBCs) on day 13th and 18th of the treatment, respectively. On the 19th, 20th, and 21st days, the animals’ delayed-type hypersensitivity (DTH) responses in each group were measured using a digital Vernier calliper, and humoral immune response was evaluated using the haemagglutination (HA) antibody technique. Blood samples were collected for Carbonic particle clearance index (K) and phagocytic index of Macrophage (α). Organ weight index (g/100g) was also recorded (α). The result showed a significant increase in the Carbonic particle clearance index (p=0.02) and phagocytic index of Macrophage (p<0.0001) in a dose-dependent manner. The study revealed a statistically significant difference in HA titres across the five groups, (p=0.02), also the DTH shows a statistically significant difference across the time (24 hours, 48 hours, 72 hours), Wilks’ Lambda = 0.291, F = 15. 86, p = 0. 0001. The aqueous leaf extract of C. occidentalis has an immunostimulatory effect on innate and adaptive immunity.

Keywords: Cassia occidentalis, innate immunity, adaptive immunity, immunostimulation, murine

INTRODUCTION

Plants have been in use for long as medicine in African subcontinents and have been an essential source of drugs, particularly in traditional medicine (Bako et al., 2005; Sumalatha et al., 2012). Immunomodulatory therapy could provide an alternative to conventional chemotherapy for various diseased conditions, especially when the host’s defense mechanisms have to be activated under the conditions of impaired immune responsiveness (Singh et al., 2015). Some plants have medicinal properties since the early stages of the development of medicine; the constant search for the medicinal plant has identified several types that have contributed to the treatment of various diseases (Sumalatha et al., 2012).

C. occidentalis is a medicinal herb commonly used in treating bacterial and fungal diseases (Ambasta, 1986). It is extensively used in the indigenous and folklore medicine system as an antidote of poisons, blood purifier, expectorant, anti-inflammatory agent, and a remedy for the treatment of liver diseases (Emmanuel et al., 2010). It is one of such frequently used African medical plants; its species are found in large number all over regions (Saidu et al., 2011), and that has long been known among the natives of the coast of West Africa, particularly in Nigeria and Ghana to be effective in the treatment of dysentery and diarrhea which are mainly caused by micro-organisms (Omoregbe et al., 1996).

The C. occidentalis roots, flowers, seeds, and leaves have been employed in herbal medicine worldwide for a variety of purposes such as purgatives, analgesic (Sini et al., 2010), and wound healing (Garba et al., 2015). The leaf extract possesses a considerable number of medicinal properties, it was found to have a diuretic and antioxidant effect (Ntchapda et al., 2015), effective for relief of dysmenorrhea (Andel et al., 2014), antibacterial and antitubercular activity (Yeragamreddy et al., 2013), improves anaemia from other diseases (Ibrahim et al., 2010), detoxification and liver protection (Yadav et al., 2010), and treatment of fevers (Singh et al., 2020). As reviewed by Verma et al. (2010), “The plant is also used to cure sore eyes, haematuria, rheumatism, typhoid, asthma, and haemoglobin disorders and was also reported to cure leprosy.” It is
commonly employed as a topical agent to heal skin conditions (Caseres et al., 1993; Ogunkunle et al., 2006).

Immune regulation is vital in maintaining normal immunity, and the search for herbal immunomodulatory compounds to treat various infections by enhancing the body’s natural resistance is of growing interest (Brindha, 2016). Immunomodulatory compounds derived from medicinal plants may be free from toxic effects; it could be safe and free from any undesirable side effect (Laemmli, 1970).

The concept of immunomodulation relates to non-specific activation of the immune system, which primarily implies a non-antigen dependent stimulation of function and efficiency of macrophages, granulocytes, complement, Natural Killer (NK) cells, lymphocytes, and also the production of various effector molecules by activated cells (Sumalatha et al., 2012). Medicinal plants need to be evaluated, given proper recognition, and developed to improve their efficacy, safety, availability, and vast application at low cost (Tilburt et al., 2008).

There is a paucity of data on the stimulatory activity of aqueous leaf extract of *C. occidentalis* on innate, humoral immune, and delayed-type hypersensitivity (DTH) responses. Our study aimed to provide vital information on the effects of aqueous leaf extract of *C. occidentalis* on macrophages’ phagocytic function, humoral immune response against SRBCs and DTH response in mice.

**MATERIALS AND METHODS**

**Plant Collection and Identification**

Fresh leaves of *C. occidentalis* were collected locally. The plant taxonomic identification was carried out at the Herbarium section of Botany Unit, Department of Biological Sciences, Faculty of Sciences, Usman Danfodiyo University, Sokoto, Nigeria. The voucher number issued was UDUS/ANS/0110.

**Preparation and Extraction**

The aqueous extract of *C. occidentalis* was collected from the Department of Immunology, School of Medical Laboratory Sciences which was initially stored and preserved at 5°C in airtight bottles and was labelled as *C. occidentalis* aqueous extracts.

**Experimental Animals**

A total of 20 mice of 8-12 weeks old, weight between 17-38 g of either sex (9 Females; 11 Males) were purchased from the Department of Veterinary Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University (ABU), Zaria, and kept in the animal house of Faculty of Pharmaceutical Sciences, Usman Danfodiyo University, Sokoto (UDUS), Nigeria. They were allowed to acclimatize for two weeks and fed with standard pelleted growers’ feed (Vital feed, Jos, Plateau) and water *ad libitum*. The Animal Ethics Committee of Faculty of Pharmaceutical Sciences, UDUS, approved the study (PTAC/Co/(AE)/OT/13-19). The experiments were conducted following regulations governing animal research.

**Animal grouping and treatment**

The animals were randomly divided into five groups of four each. Group I received 10ml/kg BW of normal saline, and Group II-V (treatment groups) received different concentrations of *C. occidentalis* (i.e., 250, 500, 1000, 1500 mg/Kg BW) respectively, orally for 21 days. At the end of the experiment all the mice survived.

**Carbon Clearance Assay**

A carbon clearance assay was used to assess the effect of the extract treatment on the Macrophage as previously described (Yin et al., 2016). Indian ink (Camel brand) suspension was diluted in a ratio of 1:50 with normal saline and used for a carbon clearance test in a dose of 0.1 ml/mice. One hour after the last treatment, the animals were weighed, then injected with 0.1 ml of Indian ink via the tail vein and timed immediately. After the ink injection, at the time of 1 min (T1) and 15 min (T2).

Blood samples were collected from orbital venous plexus using heparinized microhematocrit capillary tubes. The blood was immediately added with 3 ml 0.1% Na₂CO₃ solution to lyse the erythrocytes, and the absorbance of the mixture was measured at 675 nm using a spectrophotometer. The absorbances were A1 (at the time of T1) and A2 (at the time of T2), respectively, using the Na₂CO₃ solution’s absorbance as a blank control. Then mice were euthanatized under anesthesia. The liver and spleen were exercised from the animal and were weighed immediately. The carbonic particle clearances index (K) and macrophage phagocytose index (α), as well as organ weight index, were calculated as below:

Carbon particle clearances index (K) = \( \log A1 - \log A2 \)

Macrophage phagocytose index (α) = \( K^{0.5} \times \text{weight} \)

Index (g/100g) = \( \frac{\text{Weight of spleen}}{\text{Body weight}} \times 10 \)

**Hemagglutination assay**

**Preparation of SRBCs (Antigen)**

SRBCs were prepared as described by Dash et al. (2006). Briefly, fresh blood sheep were collected from a local slaughterhouse in freshly prepared sterile Alsever’s solution in a 1:1 ratio. Then centrifuged at 2000 rpm for 10 minutes and washed with physiological saline 4-5 times. The cells were suspended in buffered saline and finally adjusted to a 1x10⁸ cells/ml concentration for sensitization and challenge.

**Antibody response to SRBCs**

On the 13th and the 18th day of the study, the mice from all the groups were sensitized and challenged, respectively, with SRBCs in normal saline (0.1 ml of suspension containing 1x10⁸ SRBCs), intraperitoneally. Blood was withdrawn (24 hours after the last dose) from the retro-orbital plexus of all antigenically sensitized and challenged mice, then centrifuged at 3000 rpm for 5 minutes to obtain serum.

**Direct Hemagglutination**
The procedure was described by Haghighi et al., (2005). Briefly, the serum sample was incubated at 56 °C for 30 min to inactivate the complement. Fifty microliters (50 µl) of Phosphate Buffered Saline (PBS) containing 0.05% Bovine Serum Albumin was dispensed into each well of round-bottomed 96-well microplate. Serum samples (50 µl) were then added and serially double diluted in the wells from columns 2 to 12. The first column (PBS only) of wells was considered as blank. Then, 50 µl of 1% SRBCs in PBS was added to all the wells to make a 100 µl final volume. Subsequently, the plates were shaken for 1 min and incubated for 24 hours at 37°C and agglutination was monitored visually. The highest dilution capable of visible agglutination was considered as the antibody titre. A positive result was recorded when at least 50% of SRBCs agglutination was observed.

Delayed type hypersensitivity reaction
On day 13, the mice were subcutaneously injected with 0.1 ml of SRBCs suspension containing 1 x 10^8 cells/ml on the right hind footpad, while the left hind footpad received 0.1 ml of normal saline only. On day 18, the right hind footpad received 0.1 ml of normal saline only (i.e., control foot). Whereas left hind footpad was challenged subcutaneously with 0.1 ml of 1 x 10^8 cells /ml SRBCs suspension (i.e., challenge foot). The thickness of the left and right hind footpads were measured at 24, 48, and 72 hours using RD DC 706 digital Vernier callipers (Raider Pro Professional Tools Co. Ltd., China). The difference in the thickness of the right hind footpad and the left hind footpad was used to measure DTH response (Doherty, 1981; Smith et al., 2010).

Statistical Analysis
The results obtained were entered into SPSS version 25 for analysis. Continuous variables were expressed as mean and standard deviation (SD) or Median. Test for normality was performed to ascertain the normal distribution of the variables. Macrophage phagocytose index and DTH were normally distributed, while Carbonic particle clearance index, Organ weight index and HA titre were not normally distributed based on tests of normality results: Shapiro-Wilk, supported by Q-Q plot. Therefore a one-way-between- groups ANOVA for macrophage phagocytose index, one-way repeated measures ANOVA for DTH and Kruskal Wallis test for carbonic particle clearance index, Organ weight index and HA titre was carried out to compare mean or median respectively. Bonferroni or Mann Whitney tests were used as post hoc tests to compare some groups. The p-value ≤ 0.05 was used to determine the level of statistical significance.

RESULTS
Effect on Carbonic Particle Clearance and Macrophage Phagocytose Indexes
As depicted in table 1, Group V recorded the highest K median scores (Md= 0.0190) while the least is group I (Md= 0.0001). A Kruskal-Wallis test revealed a statistically significant difference in K across the five groups (χ^2 =11.52, p=0.02). A Post-hoc test using the Mann-Whitney U test revealed a significant difference in K between Group I vs. Group IV (p=0.04), and Group I vs. Group V (p=0.04). Again, on macrophage phagocytose index, Group V recorded the highest mean scores (M: 1.87, SD: 0.12) while the least is a group I (M: 0.37, SD: 0.03). There was a statistically significant difference in Macrophage phagocytose index across the groups after treatment with C. occidentalis (p<0.0001). Post-hoc comparisons indicated that the macrophage phagocytoses index mean score for normal control was significantly different from group II (p<0.0001), group III (p=0.005), group IV (p<0.0001), and group V (p<0.0001).

Table 1: Effect of aqueous leaf extract of C. occidentalis on Carbon clearance and Macrophage Phagocytose indexes in Mice

<table>
<thead>
<tr>
<th>Groups (N=20)</th>
<th>Dose (kg BW)</th>
<th>Carbon clearance index</th>
<th>Macrophage Phagocytose index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>χ^2</td>
</tr>
<tr>
<td>I (Normal saline)</td>
<td>10 ml</td>
<td>0.0001</td>
<td>c,d</td>
</tr>
<tr>
<td>II (C. occidentalis)</td>
<td>250 mg</td>
<td>0.0002</td>
<td>c,d</td>
</tr>
<tr>
<td>III(C. occidentalis)</td>
<td>500 mg</td>
<td>0.0002</td>
<td>0.87 (0.19)</td>
</tr>
<tr>
<td>IV(C. occidentalis)</td>
<td>1000 mg</td>
<td>0.0190</td>
<td>1.26 (0.10)</td>
</tr>
<tr>
<td>V (C. occidentalis)</td>
<td>1500 mg</td>
<td>1.87 (0.12)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

a= grp I vs II, b= grp I vs III, c= grp I vs IV, d= grp I vs V, *p<0.05, **p<0.006, ***p<0.0001

Effect on Organ Weight Index
From table 2, Group V recorded the highest organ weight index median score (Md= 0.0410) while the least is group I (Md= 0.0001). A Kruskal-Wallis test revealed a statistically significant difference in organ weight index (g/100 g) across the five groups ($\chi^2 = 12.63, p=0.01$). A Post-hoc test using the Mann-Whitney U test revealed a significant difference in organ weight index between Group I vs. Group IV ($p=0.04$) and Group I vs. Group V ($p=0.04$).

**Table 2: Effect of aqueous leaf extract of *C. occidentalis* on organ weight Index in Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>N=20</th>
<th>Dose (Kg BW)</th>
<th>Organ weight index</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Normal saline)</td>
<td>10ml</td>
<td>0.0001</td>
<td>$\chi^2$</td>
<td>0.01</td>
</tr>
<tr>
<td>II (C. occidentalis)</td>
<td>250 mg</td>
<td>0.0020</td>
<td>12.63</td>
<td></td>
</tr>
<tr>
<td>III (C. occidentalis)</td>
<td>500 mg</td>
<td>0.0090</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV (C. occidentalis)</td>
<td>1000 mg</td>
<td>0.0099</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V (C. occidentalis)</td>
<td>1500 mg</td>
<td>0.0410</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a= grp I vs. IV, b= grp I vs. V, *p<0.05

**Effect on Humoral Immune Response against Sheep Red Blood Cells**

As shown in Table 3 below, the HA titre of the Mice receiving the highest dose (group V) of the plant extract recorded a highest median score (Md = 64) while those in group I and II recorded the least median scores. A Kruskal-Wallis test revealed a statistically significant difference in HA titres across the five groups ($\chi^2 =12.26, p=0.02$). A Post-hoc test using the Mann-Whitney U test revealed a significant difference in HA titre between Group I vs. Group III ($p=0.03$), Group I vs. Group IV ($p=0.04$), Group I vs. Group V ($p=0.04$).

**Table 3: Effect of aqueous leaf extract of *C. occidentalis* on humoral immune response in mice**

<table>
<thead>
<tr>
<th>Groups (N=20)</th>
<th>Dose (Kg BW)</th>
<th>HA titre Median</th>
<th>$\chi^2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Normal saline)</td>
<td>10ml</td>
<td>8</td>
<td>12.26</td>
<td></td>
</tr>
<tr>
<td>II (C. occidentalis)</td>
<td>250 mg</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III (C. occidentalis)</td>
<td>500 mg</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV (C. occidentalis)</td>
<td>1000 mg</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V (C. occidentalis)</td>
<td>1500 mg</td>
<td>64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a= grp I vs III, b= grp I vs IV, c= grp I vs V, *p<0.05

**Effect on Delayed-Type Hypersensitivity response**

The DTH response at 72 hours recorded a highest mean difference (SD) 1.46 (0.89) whereas DTH response at 24 hours recorded the least mean difference. A one-way repeated measures ANOVA was conducted to compare the DTH response between 24 hours, 48 hours and 72 hours. There was a significant statistical difference across the time (24 hours: M= 0.33 mm, SD= 0.10), (48 hours: M= 0.44 mm, SD= 0.11) and (72 hours: M= 1.46 mm, SD= 0.89), Wilks’ Lambda = 0.291, F = 15.86, p = 0.0001. A Post-hoc comparisons using the Bonferroni tests indicated that the mean DTH response at 24 hours (M = 0.33 mm, SD = 0.10) was significantly different from that at 48 hours (M =0. 44 mm, SD = 0.11), p = 0.007 and that at 72 hours (M = 1. 46 mm, SD = 0. 89), p= 0.0001. So also mean DTH response at 48 hours (M =0. 44 mm, SD = 0.11) was significantly different from that at 72 hours (M = 1. 46 mm, SD = 0. 89), p = 0.001 (Table 4).

**Table 4: Comparison of the mean difference of DTH response at 24, 48, and 72 hours**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>DTH (mm)</th>
<th>Wilks’ Lambda test</th>
<th>Post hoc test</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean difference (SD)</td>
<td>Value</td>
<td>p-value</td>
<td>Time comparison (hr)</td>
<td>p-value</td>
</tr>
<tr>
<td>24</td>
<td>0.33(0.10)</td>
<td>0.29</td>
<td>0.0001</td>
<td>24 vs. 48</td>
</tr>
<tr>
<td>48</td>
<td>0.44(0.11)</td>
<td>0.29</td>
<td>0.0001</td>
<td>24 vs. 72</td>
</tr>
<tr>
<td>72</td>
<td>1.46(0.89)</td>
<td>0.29</td>
<td>0.0001</td>
<td>48 vs. 72</td>
</tr>
</tbody>
</table>

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DISCUSSION

Medicinal plants have been used as an alternative to orthodox medicine in many countries, Nigeria inclusive. Despite their widespread use, few scientific studies have been undertaken to ascertain the efficacy of traditional remedies (Graça et al., 2017). Thousands of plant species have been identified as a good source of therapeutics, and the use of these compounds is gradually increasing (Ishii et al., 1984).

In the present study, *C. occidentalis* showed a significant increase in carbonic particle clearance (K) and macrophage phagocytosis index (α) in the dose-dependent increase suggesting the *C. occidentalis* stimulatory effect on macrophages. Macrophages are professional antigen-presenting cells that phagocytose pathogens and debris as innate immunity effector cells. Moreover, macrophages process and present the antigens they recognise and engulf to CD4+ helper T cells to initiate an adaptive response, thereby bridging innate and adaptive immunity (Gokhale et al., 2003).

The current study findings are in line with previous studies (Anarthe et al., 2014; Bin-Hafeez et al., 2003; Vikasari et al., 2015). The Stimulatory activity of *C. occidentalis* on immune cells may be due to the presence of various Phyto-constituents present in the extract like quinines, flavonoids, tannins, and alkaloids, which are already reported to possess immunomodulatory activity (Anarthe et al., 2014). Several studies reported the immunostimulatory effect of plants: Methanolic extract of *Swietenia mahagoni* seeds (Hajra et al., 2012), an aqueous extract of Tridax (Tiwari et al., 2004), an aqueous extract of seeds of *Abrus precatorius* Linn (Tilwari et al., 2011).

The carbon clearance assay shows the rate at which carbon particles are removed from blood after systemic injection of carbon particle in the form of an ink, and the rate of the clearance was deduced mathematically through a given equation (Gokhale et al., 2003). The removal of carbon particles was made by phagocytic cells such as macrophages. At the same time, macrophage activity were assessed by the assay (Ponkshe et al., 2002). The phagocytic process defect may lead to several disease conditions in humans (White et al., 1986). Because macrophages are essential in the secretion of several cytokines, it ultimately leads to the activation of other immune cells (Tilwari et al., 2011).

*C. occidentalis* showed a significant increase in organ weight index (g/100g) at higher dose. This implies the plant extract may potentially have concentration dependant effects on the organ weight. Several studies are in line with our finding, Hajra et al. (2012) on Methanolic extract of *Swietenia mahagoni* seeds, Anarthe et al. (2014) on water extract of *Stachytarpheta jamaicensis* L. in albino rats, and Tiwari et al. (2004) on aqueous extract of *Tridax procumbens*. However, in contrary to this study findings Vikasari et al. (2015) reported a significant decrease in organ weight of rats after treatment with Methanolic extract of *Trigonella foenum graecum* whole plant and also that of Tilwari et al. (2011), who reported that immunomodulatory activity of the aqueous extract of seeds of *Abrus precatorius* Linn.

The humoral immune response against SRBCs antigen reveals a significant increase in HA antibody titres with an increase in plant extract concentration; this implies treatment with the formulation may have the ability to activate lymphocytes, especially the B lymphocytes and other cells associated with humoral immune response. Antibodies are produce by plasma cells upon the interaction between B lymphocytes and antigen thus lead to its proliferation and differentiation into the antibody secretion cells. The antibodies produced were set to bind to the antigen and neutralised or facilitated recognition by phagocytes; this leads to the ultimate elimination of the antigen (Fulzele et al., 2003; Gokhale et al., 2003).

Several studies reported a significant increase in circulating antibodies after treatment with different plant extract, *Aegle marmelos* (Patel et al., 2010), *Acacia polyacantha* wild (Ismail et al., 2009), *Adensonia digitata* (Rahul et al., 2015), *Argyreia speciosa* root (Gokhale et al., 2003), and *Swietenia mahagoni* (Hajra et al., 2012). However, Tilwari et al. (2011) reported inhibitory effect on humoral immune response in mice after treatment with *Abrus precatorius*. The difference may be attributed to suppression of functional activity of antigen presenting cells or type and level of cytokines present at the microenvironment after activation of lymphocytes (Allison, 2000).

This study reported no significant difference between the normal control group and group II (a group that received a low dose of the extract). The indifference may be attributed to the extract’s small dosage, which may not significantly modulate the immune response as a low dose might bring about little or no detectable immune response against SRBCs antigen between the groups. Mirabeau et al. (2012) reported a much higher immune-stimulating effect in the presence of a higher plant extract dose. Of note the Lethal dose (LD50) of our extract was 5000 mg/kg BW using Lorke’s method (Lorke, 1983).

After 24 hours, the result on DTH did not show a significant increase, but after 48 hours, the result shows a significant increase, and a more robust increase in the thickness of the footpad was observed after 72 hours. The above suggests that the plant has immunostimulatory activities on DTH response. The increment may be attributed to the ability of the Extract to activate lymphocytes and other immune cells, thereby increasing cell-mediated immunity.

In DTH response, activated T cells activate and recruit more macrophages through secretion of lymphokines, which increases vascular permeability, induces vasodilatation, and produces inflammation (Nmami et al., 2015). Previous studies (Kumolosasi et al., 2018; Manjuladevi et al., 2013; Ramesh et al., 2016) also reported an increase in DTH response after treatment with plant materials. Further study will be required.
to include relevant control groups to improve on the credence of this study findings.

CONCLUSION

The aqueous leaf extract of Cassia occidentalis has immunostimulatory activities on macrophage phagocytic function, humoral and cell-mediated immune responses in a dose-dependent manner. In brief, Cassia occidentalis inherent ability to induce activation of lymphocytes involved in humoral and cell mediated immune responses indicates the immunomodulatory potential of the extract and in-depth research needed to ascertain our finding.

Conflict of Interests

The authors declare no conflict of interest.

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