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In vitro Bioactivities of Aqua Alcoholic Extracts of Plant *Boerhavia Diffusa Linn*.

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ABSTRACT

Background: *Herbal extract of various plants have been reported to have cytotoxic and antioxidant effects. However, nanoparticle synthesis from these plants requires high resources and technical expertise. There is a need for a cost effective Nano formulation preparation method. Boerhavia diffusa is an herbal plant used for its cytotoxic and antioxidant effects in various diseases. The objective of the present study is to evaluate the in vitro antioxidant and cytotoxic activity from the aqua alcoholic extracts of plant Boerhavia diffusa.*

Materials and Methods: The current study was a cross-sectional observational analytic study. Our study includes 150 children having the age group of 3 to 12 years who visit the Department of Pediatric Dentistry in the College of Dentistry, King Khalid University. One hundred and fifty children (range 3-12 years) were included whose parents reported snoring on a regular basis and signs or symptoms of OSA. A self-administered questionnaire was prepared in both Arabic and English languages and distributed to all the participants. All the data were collected and analyzed by using SPSS version 21.

Materials and Methods: Assessment of antioxidant activity is carried out by in vitro method Diphenyl-1-picryl hydrazyl radical (DPPH) scavenging method and cytotoxic activity live brine shrimp assay by counting the number of live brine shrimps after 24 hours and 48 hours. Values are tabulated and spearman correlation analysis was done in SPSS software version 23.

Results: The activity of the aqua alcoholic extract was compared with standard ascorbic acid by measuring absorption intensity in the spectrophotometer at the wavelength of 517 nm. While increasing the concentration of plant extracts the percentage of DPPH inhibition also increased which showed positive correlation(r=1). As a result of the death of numerous nauplii, cytotoxic activity increased, which was measured after 48 hours and it shows negative correlation(r=-1).

Conclusion: Boerhavia diffusa have cytotoxic and antioxidant properties. The natural plant based nanoparticles with its phytoconstituents in a single pace adds up a huge advantage in eco-friendly nano research.

Key words: *Boerhavia diffusa*, Antioxidant, Cytotoxicity, Aqua alcoholic extract, Innovative technique

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Background

Boerhavia diffusa (Punarnava), belongs to the family Nyctaginaceae with forty species. Among these species, *Boerhavia diffusa* is widely distributed in subtropical and tropical areas of Africa, Asia, America, and Australia [1]. Among all these, *Boerhavia diffusa* is the most widely studied plant and has many uses in Ayurvedic and Unani medicines [2]. In Ayurveda, this medication is utilized as Sothaghna (Anti-inflammatory), Mootrala (diuretic), Kasahara (Antitussive), Rasayana (rejuvenator), jwarahara (antipyretic) [3]. The root has numerous therapeutic benefits in the treatment of diabetes, inflammation, jaundice, spleen enlargement, stress, dyspepsia, stomach discomfort, heart disease and bacterial infections. Some tribal cultures eat the entire plant of this herb [4,5]. It has also been reported to be effective as an antiviral agent and also for the treatment of elephantiasis, night blindness, corneal ulcers, and various liver disorders [6]. It has been commonly used in Nigerian traditional medicine for treating epilepsy [7] miscarriage, and menstrual pain [8]. The secondary metabolites of *Boerhavia diffusa* hydroethanolic extract had different medical benefits such as elevated antioxidant activity providing some support for cardiovascular and weight reduction [9]. Chemical components and medicinal activity of *Boerhavia diffusa* have been extensively researched. Rotenoids, flavonoids, flavonoid glycosides, xanthones, purine nucleoside, lignans, ecdysteroids, and steroids are some of the isoflavones found in the roots.

Various animal studies and trials have confirmed the presence of activities, for example, immunomodulation, hepatoprotection, antifibrinolytic, anticancer activity, antidiabetic activity, anti-inflammation, and diuresis [10,11]. An important indication of BD in traditional medicine is abdominal tumor. Various studies (in vitro and in vivo) suggest the presence of potential anticancer compounds in various extracts prepared from various plant parts. Manu and his colleagues extracted the alkaloid punarnavine from the roots and discovered that it has antimetastatic properties [12]. Similarly, Srivastava and coworkers showed a dose-dependent in vitro cytotoxic effect of the extract of the *Boerhavia diffusa* root and the leaf in HeLa and U-87 tumor cell lines. Crude ethanolic extract of the root (200µg/mL) and the leaf (300µg/mL) showed 30 and 40% cell death while alkaloidal fraction (300µg/mL) and methotrexate (200 nM) showed 40% cell death [13,14]. Studies showed that Boeravinone, which is also present in Punarnava, also shows antioxidant and cytotoxic activity [12,15]. Rachh and coworkers evaluated ethanolic and methanolic extracts of the dried root powder for antioxidant activity. The extract showed good in vitro antioxidant activities in terms of ferric reduction and hydrogen peroxide quenching in comparison to ascorbic acid [16].

But recent research suggests that antioxidants decrease the risk of chronic diseases including heart disease and cancer [17]. Natural methods like herbal medicines play a vital role in the prevention and treatment of cancer. A significant number of studies on photochemistry, pharmacology have been carried out with no sufficient data available on in vitro activities. Our team has extensive knowledge and research experience that has translated into high quality publications [18-37]. The present study aimed to study the antioxidants and cytotoxic activity of aqua alcoholic extract of *B. diffusa*.

MATERIALS AND METHODS

Study setting

The present in vitro study was conducted in the Nano Research lab in Saveetha dental college and hospitals Chennai. The powdered form of *Boerhavia diffusa* plant extract was readily available in customized form obtained from Botanical supplier's Chennai. The readymade availability of plant excerpts adds an advantage in minimizing the manual chores and time.

Nanoformulation of plant extract

Fresh dried and powdered readily available *Boerhavia diffusa* plant material of 1gm is measured in an unbiased manner along with 100ml of distilled water and filtered with Whattman filter paper. 10ml of ethanol is mixed with the clear filtered extract. The (tortilla) colour change is observed. The validation of the procedure is finalized by the principal investigator and senior researchers (Figure 1).

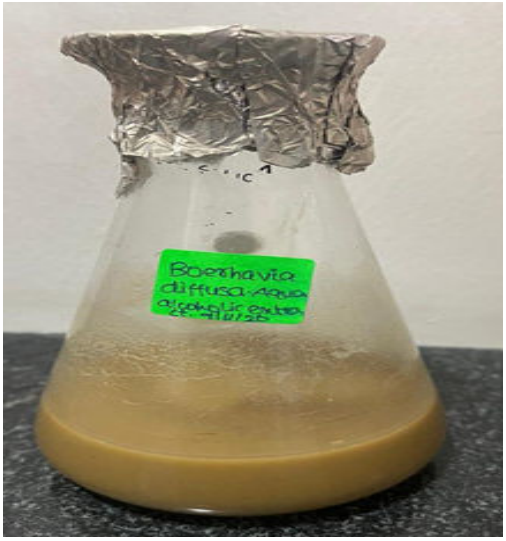

Figure 1: Filtered extract of *Boerhavia diffusa*.

Reagent and chemicals

DPPH (400 µg/mL),

5 mL of solutions of aqua alcoholic *B. diffusa* extracts of different concentrations (10 to 50 µg/mL)

Positive control: ascorbic acid, potassium dichromate

Negative control: DMSO: dimethyl sulfoxide

Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging method

The radical scavenging activities of aqua alcoholic extract of *Boerhavia diffusa* were estimated using stable free radicals of DPPH.

About 2.0 mL of methanol solution of each extract at different concentrations (10, 20, 30, 40 and 50 µg/mL) were mixed with 3.0 mL of DPPH methanol solution (20 µg/mL).

After an incubation period of 30 min, the absorbance was measured at 517 nm against methanol as blank by using a UV-visible spectrophotometer. The radical scavenging activity (%) was calculated based on the following formula:

% Inhibition=OD of control-OD of test/OD control*100

The percentage scavenging activity of each extract was compared with ascorbic acid, the positive control.
IC50 value of each extract was determined from the plotted graph of percentage DPPH neutralization and concentration of extract, which was defined as the amount of antioxidant required to reduce the initial DPPH free radical concentration by 50% (Figure 2).

Figure 2: Evaluation of antioxidant activity by DPPH assay.

Cytotoxic activity assay

Brine shrimps are zooplankton adult habitats of Nauplii. The amount of lethality of the principle extract solution of *Boerhavia diffusa* was analysed from the number of live brine shrimp in room temperature at different concentrations (10-50µl) of the prepared bio extract loaded in the microplate.

Twelve well microplates are taken. The appropriate amount of extracts was dissolved in a particular volume of pure dimethyl sulfoxide (DMSO) to make stock solutions for the samples. 4ml of distilled water is given to each well.

The five different concentrations of plant extract are added to the well. The labelling of the concentrations are given to avoid errors. Salt water with DMSO is added up as negative control.

Ten Adult brine shrimps are pipetted into each vial. It was kept undisturbed for 24hours. The number of live shrimps was counted after 24 hours and 48 hours. The cytotoxic effect has been evaluated by counting the number of live shrimps in the well.

The different readings are then labelled. The values obtained from antioxidant and cytotoxic activity assay are entered in excel sheets and spearman correlation analysis was done statistically with IBM SPSS software version 23 (Figure 3).

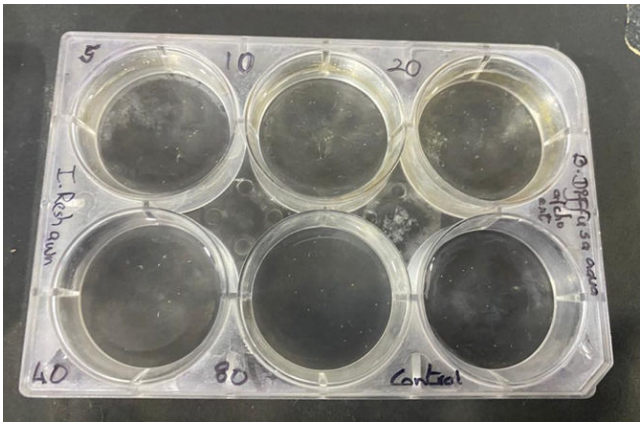

Figure 3: Cytotoxicity effect analyzed by several brine shrimps in microplate after 48 hours of incubation time.

RESULTS

Antioxidant activity

The antioxidant activity of *Boerhavia diffusa* aqua alcoholic extract was determined by DPPH activity. The activity of the aqua alcoholic extract was compared with standard ascorbic acid by measuring absorption intensity in the spectrophotometer at the wavelength of 517 nm. While increasing the concentration (10 µL, 20 µL, 30 µL, 40 µL, 50 µL) of plant extracts, the percentage of inhibition of DPPH inhibition also increased. However, the inhibition percentage is directly proportional to the concentration of leaf extract as shown in **Figures 4-5**. Spearman correlation analysis showed positive correlation(r=1) with significant p value of less than 0.05. The absorption intensity with different concentration was tabulated in **Table 1.**

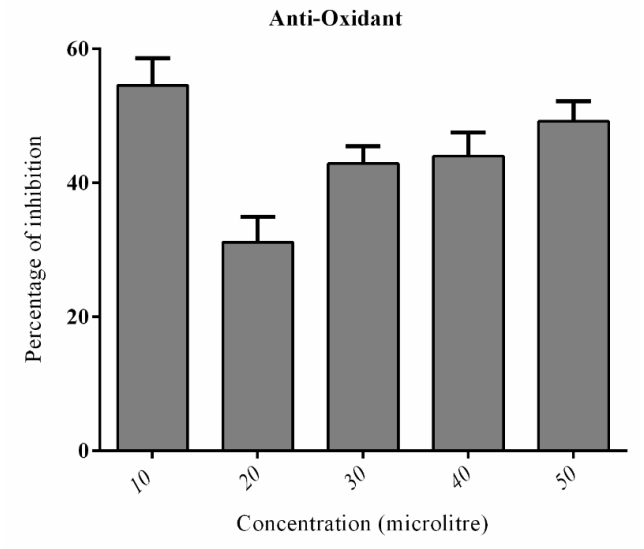

Figure 4: *Boerhavia diffusa* showed increased DPPH inhibition with increase in concentration of extract. Inhibition was measured after 48 hours of incubation. X axis shows concentrations in microliter, Y axis shows percentage of inhibition. Values are expressed as the mean percentage of inhibition which shows positive correlation(r=1) at varying concentration.

Table 1: the observed values are tabulated with different concentration and corresponding absorbance.

Concentration	Wavelength	Absorbance	%t to absorbance formula
10	517	0.229	35.4
20	517	0.22	35.4
30	517	0.36	43.7
40	517	0.33	46.1
50	517	0.45	57.7

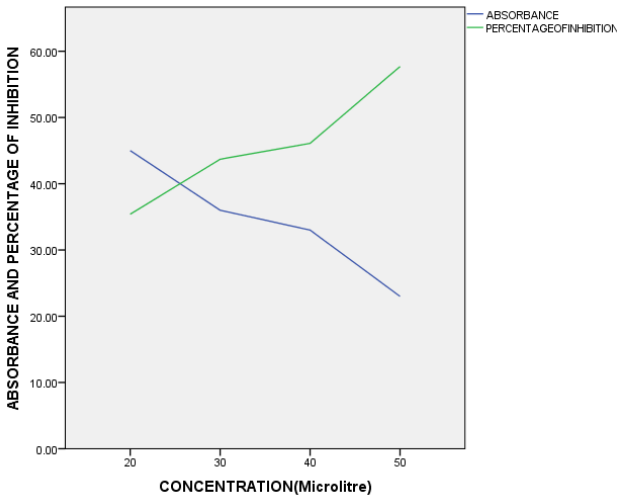

Figure 5: Line graph showing decrease in absorbance showed an increase in the percentage of inhibition.

Cytotoxic activity

Brine shrimp lethality assay was performed using the formulated solution. The results obtained from the assay are tabulated and converted into a bar graph as Figure 6 for easy representation. The X-axis represents the concentration of the formulated solution used. The Y-axis represents the number of live nauplii. The graph explains the positive correlation of cytotoxic activity with concentration and the number of live brine shrimps. The formulated solution is given to the nauplii for two days at different concentrations.

At 5 µl concentration, all 10 nauplii are alive, at 10 µl of solution 10 nauplii were alive at day 1 and 9 nauplii were alive at day 2. Then the solution is given at 20 µl concentration, 10 nauplii are alive on day 1 whereas 9 nauplii were alive on day 2. At 40 µl concentration, only 8 nauplii were alive on day 2.

At 80 µl concentration, only 4 nauplii were alive. In the test control, no extract is added, and though all the nauplii are alive for both the days. On day 2, Decreases in the number of nauplii counts with increase in concentration are represented in Figure 7 and relation between control group and test group are shown in Figure 8.

Spearman correlation analysis showed negative correlation($r=-1$) with p value<0.05. Results were tabulated with different concentrations of day 1 and day 2 in Table 2.

Table 2: The number of live nauplii at different concentration of extract after 24hours and 48 hours in relation to the control.

Concentration	5 µL	10 µL	20 µL	40 µL	80 µL	Control
Day 1	10	10	10	10	10	10
Day 2	10	9	9	8	4	10

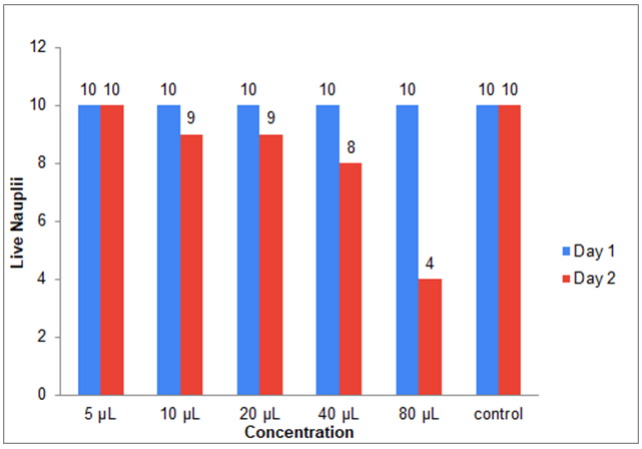

Figure 6: Cytotoxic activity of *Boerhavia diffusa* aqua alcoholic extract on live nauplii. Live nauplii were treated with 5 µL, 10 µL, 20 µL, 40 µL, 80 µL and control (Ascorbic acid) and counts were measured on day 1 and day 2. It showed a decrease in the number of live nauplii counts with the concentration (microliter) Values are expressed as total count.

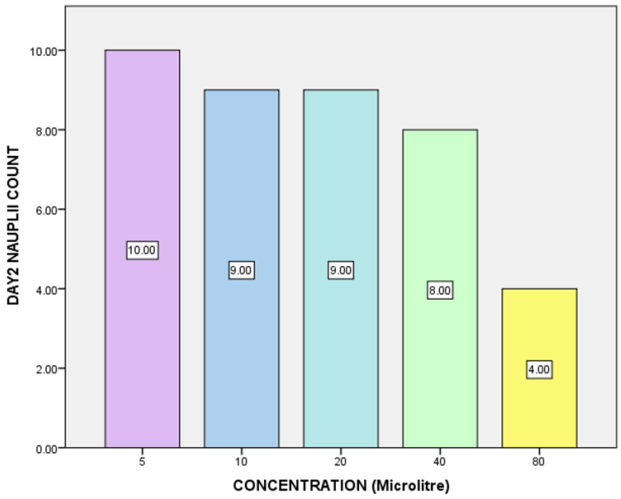

Figure 7: Cytotoxic activity of *Boerhavia diffusa* aqua alcoholic extract showed decrease in the number of live nauplii count with the concentration (microlitre) which shows negative correlation(r=-1).

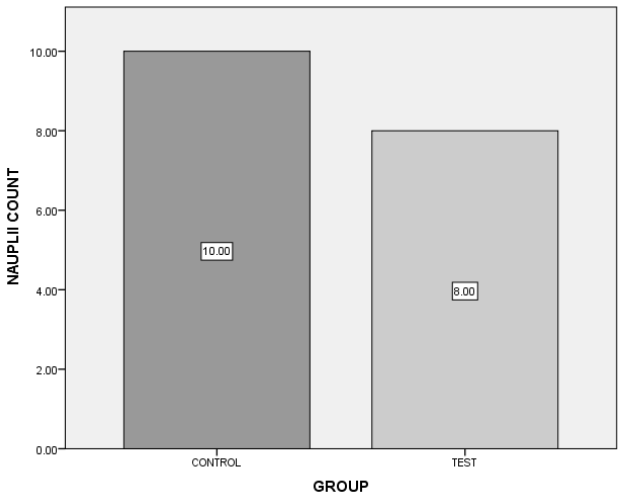

Figure 8: Test group showed a decrease in live nauplii count

DISCUSSION

Boerhavia diffusa methanol extract antioxidant activity was observed dependent on the scavenging potential of the free radicals (DPPH, NO) due to the presence of flavonoids which are Polyphenolic compounds in methanol extract [38]. The results agree with other researchers' findings [39] about the antioxidant role of methanol extract *B. diffusa* has been identified as a result of the presence of rotenoid (boeravinone G, H, and D). Boeravinone G plays a significant role in rotenoids and is also a Polyphenolic agent [40]. Powerful antioxidant activities against free radical DPPH have been demonstrated by various solvent-derived seed extracts. In this study, the increased concentration of all the extracts corresponded to an increase in their antioxidant properties [41]. Previous literature study on antioxidant activity of chitosan nanoparticles and chitosan-lycopene Nano composite solution has increased activity when compared to the standard ascorbic acid, so lycopene mediated chitosan nanoparticles have the potential to be used as an effective antioxidant agent. The synthesis of metal nanoparticles and Nano composites is an emerging area of research and exploration in the field of material science for their unique size and shape [42]. Lycopene with an increase in concentration has increased antioxidant activity but was comparatively less when compared to the standard ascorbic acid. Similarly, silver nanoparticles, graphene oxide nanoparticles and GO-Ag Nano composites were synthesized from seed extracts of amla fruit which showed a good antibacterial effect against oral pathogens with minimal cytotoxicity [43]. Most recently concluded that aqua alcoholic extract of grape-seed proved to be a potent antioxidant and antimicrobial agent and can therefore be used for application in the medical field [44]. A similar study was undertaken to evaluate the antioxidant activity of Ethanol, Chloroform, and Ethyl acetate fraction of *Boerhaavia diffusa L* roots which might have improved its hepatoprotective action. In Vitro nitric oxide scavenging activity, the percentage inhibition was 71.35%, 33.74%, 23.85% in ethanol, chloroform, and ethyl acetate extracts at 250mcg/ml and this was compared with curcumin at 62 mcg/ml showed only 84.7% inhibition respectively. Extract of chloroform showed a dose-dependent increase and dual-phase response in ethyl acetate and ethanolic extract. In DPPH radical scavenging activity, the ethanol extract showed 81.94% inhibition and the chloroform extract showed 42.58% inhibition at 1000mcg/ml compared with 88.02 % inhibition by Quercetin. The above results suggest that roots of *Boerhaavia diffusa* were found to reveal antioxidant potential which supports the use of this plant in traditional medicine [45,46] Ethanol extract of *Boerhavia diffusa* extract inhibits gram-positive bacteria such as *S. aureus*, *B. subtilis, S. faecalis*, and *M. luteus* as well as all gram-negative bacteria used in this study [47]. Except for *M. luteus* and gram-negative bacteria such as *K. pneumoniae*, *P. vulgaris*, *S. marcescens*, and *S. flexneri*, methanol extract had an inhibitory effect against all gram-positive bacteria tested. P. aeruginosa had the highest susceptibility in aqueous extract, followed by S. aureus and E. coli had the lowest susceptibility. The antimicrobial activity of the various extracts increased as the concentration was increased. The findings of the research back up local practitioners' ethnomedicinal use of this herb. The findings of this study revealed that both aqueous and ethanolic extracts of *B. diffusa* had antibacterial activity on E. coli, S. aureus, and P. aeruginosa [48]. Furthermore, studies have shown that the aqueous form of the drug (2 ml/kg) has greater hepatoprotective efficacy than the powder form, which is likely due to the liquid form's stronger absorption through the intestinal tract. The hepatoprotective function of *Boerhaavia diffusa L*. roots was found to protect serum parameters in rats suffering from thioacetamide toxicity [49]. Another study shows that the aqueous extract of thin roots collected in the summer has a higher operation, indicating that root collection should be done at the right time and with the right form of roots for the best results which also

supports the usage of *B. diffusa L*. roots by many Indian tribes for hepatic ailments [50].

About cytotoxicity, a study stated upon this mitosis of *C. jagus* roots, a *B. diffusa* extract had a strong depressive effect. The study used *B. diffusa* extract, and the control experiment's mitotic index was found to be 5.27. The concentrations of the treatment extracts and the mitotic indices derived from their operation had a negative association. This indicates that this extract inhibits mitosis. The mitotic index was substantially inhibited as the concentration of *B. diffusa* treatment solution was increased. This time, the concentration of the extract and the mitotic indices provided by the observed behavior have a very negative correlation. Since the root extracts of *B. diffusa* have the potential to accumulate metaphase and therefore inhibit mitosis, they can be used instead of the more costly colchicine for cytological studies [51]. In the previous study, from the findings (higher LC50 values than 100 µg/mL) of brine shrimp lethality bioassay, it can be concluded that the extracts of *B. diffusa* do not show any apparent in vitro toxicity compared to positive control which supports the tribes' use of the plant as a non-toxic leafy vegetable. Moreover, the vegetable was found by the researchers as a rich source of some macro and micronutrients [52]. Significant amounts of alkaloids are also non-carcinogenic to the plant [1]. Studies have shown that leaves have nutritional components that can be considered an effective part of the diet, such as vitamin C and calcium.It is therefore quite well assumed that *Boerhavia diffusa* hydroethanolic extract plays an important role in the scavenging of free radicals and serves as a possible source of natural antioxidants that may be responsible for the existence of secondary metabolites such as phenols, flavonoids, tannin, and alkaloids so *Boerhavia diffusa* may also act as a potent natural medicine [3].

This study with *Boerhavia diffusa* aqua extract with the methanolic and ethanolic extract was taken into consideration. Future study has to be elaborated under increased concentration and larger sample size of other species of family Nyctaginaceae. Extract must be tested on various cell lines to check its effectiveness, compatibility and active compounds which exhibit antioxidant properties might be isolated and formulated with other herbal products.

CONCLUSION

Boerhavia diffusa have cytotoxic and antioxidant properties. The natural plant based nanoparticles with its phytoconstituents in a single pace adds up a huge advantage in eco-friendly nano research.

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CONFLICT OF INTEREST:

The authors would like to declare no conflict of interest in the present study.

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