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POTENT ANTICANDIDAL ACTIVITY OF THE METHANOLIC EXTRACT OF *WEDELIA CHINENSIS* LEAVES (OSBECK) AGAINST PATHOGENIC *CANDIDA ALBICANS*

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ABSTRACT

The anticandidal activity of the methanolic extract of *Wedelia chinensis* leave was studied and tested against four pathogenic *Candida albicans* strains of clinical isolates. The extract exhibited favorable anticandidal activity with the zones of inhibition between 18.00 ± 0.10 to 20.00 ± 0.17 mm and minimum inhibition concentration (MIC) values between 3.13 and 6.25 mg/mL. The time-kill studied of the extract against the selected strain, *C. albicans* 1 suggested that the extract possessed yeastocidal properties at higher concentrations and eradicated the growth of yeast cells. The SEM and TEM micrographs exhibited major abnormalities occurred on the yeast cells after exposed to the extract were complete alterations in their morphology and collapsed of the cells beyond repair. The methanolic extract of *W. chinensis* may be an effective anticandidal agent to treat pathogenic yeast infections such as candidiasis.

KEYWORDS: ***Wedelia chinensis*, anticandidal activity, minimum inhibition concentration, yeastocidal, candidiasis**

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INTRODUCTION

Fungal infections remain a significant cause of morbidity and mortality despite advances in medicine and the emergence of new antifungal agents. Candidal infection represents one of the most rapidly increasing healthcare infections with a significant mortality rate in hospitalized patients [1]. These fungal infections are becoming more prevalent worldwide because the size of the immune-compromised patient population is rising, and despite appropriate anti-fungal therapy, mortality from candidemia is over 30% [2, 3].*Candida* species are now recognized as major agents of hospital acquired infection [4]. These species are the most common fungal pathogens of humans and they are causative agents of oral and vaginal candidiasis, giving rise to severe morbidity in millions of individuals worldwide [5-8]. *Candida albicans* which is a diploid fungus that grows both as yeast and filamentous cells is the organism most often associated with serious fungal infection and it is showing increased resistance to existing antifungal agents [1, 9]. It is a common commensal of the gastrointestinal and urogenital tracts of human and it is also the cause of vaginitis in genital infections of women [10, 11]. In fact, it is one of the leading causes of opportunistic fungal infections in immune-compromised individuals, including AIDS patients, transplant recipients, and cancer patients [2, 12]. The remedial uses of commercially available antifungal drugs have induced varieties of toxic side effects [13-15]. Therefore, there is a distinct need for the discovery of new, safer, and more effective antifungal agents. Plant derived medicines have been part of traditional health care in most parts of the world for thousands of years, and nowadays there is increasing interest in plants as sources of agents to fight microbial diseases [16, 17]. Asteraceae is the largest family of flowering plants in terms of number of species. Several plants of this family are edible and are used as folk medicines, one of them is *W. chinensis* (Osbeck) Merrill which is also commonly known as “Pilabhamgara” or “Bhringraj’ in Hindi. It is also a reputed herbal medicine in both Ayurvedic and Unani system of medicine [18]. Extracts from the dried or fresh leaves of this plant are applied as a paste on wounds in some rural communities. Some work on the wound healing activity of the aqueous extract of the leaves of this plant on open wound and sutured wound models is already on record [19, 20]. The fresh juice from the leaves of *W. chinensis* has been used by Ayurvedic physicians in India for external use to treat skin problems, dermatitis, eczema and acne [21]. The herb contains wedelolactone and demethylwedelolactone (Coumestans derivatives) possessing potent anti-hepatotoxic effect and is incorporated as a major ingredient in a number of developed potent anti-hepatotoxic phytopharmaceuticals formulations [22, 23]. It is useful in the treatment of osteoporosis of knee and also possesses anti-inflammatory, antibacterial and antifungal activities [24, 25.] Therefore, the present work is carried out to evaluate the anticandidal activity of the methanolic extract of *W. chinensis* leaves on four pathogenic *C. albicans* strains. The effects of extract on the candidal cell growth and cell morphology were studied and investigated.

MATERIALS AND METHODS

(i) Collection, processing and extraction of plant sample

The fresh sample of *W. chinensis* leaves was collected around the Penang Island and brought back immediately to the laboratory in a sterile plastic bags. The leaves were rinsed thoroughly under running tap water and the clean samples were then dried in an oven at 45°C for 4-7 days until they were completely dried before grinding them into powder form. Approximately 40 g of dried powder form of the plant sample was soaked in 400 ml of 100% methanol at room temperature (30±2°C) for three consecutive days with frequent agitation. The mixture was filtered using a muslin cloth and followed by Whatman No. 1 filter paper. The filtrate was then concentrated in a rotary evaporator under reduced pressure until oily paste formed and kept at cool dry place until further used.

(ii) Microorganisms and cultural maintenance

Four pathogenic *C. albicans* strains which were obtained from the Industrial Biotechnology Research Laboratory Culture Collection, School of Biological Sciences, Universiti Sains Malaysia were used throughout the study. The *C. albicans* strains were designated as 1, 2, 3 and 4 which were previously isolated from patients with urinary tract infection, vaginitis, yeast onychomycosis and skin infection, respectively. The cultures were maintained on Sabouraud dextrose agar slants at 37°C for 48 hours. All the cultures were kept at 4°C until further used. Subculturing was done at every four weeks to maintain their viability.

(iii) Anticandidal activity by disc diffusion method

The anticandidal activity of the extract against the test pathogenic yeast strains were determined following the method described by NCCLS [26] with slight modifications. The yeast strains were removed aseptically with an inoculating loop and transferred to test tubes containing 5.0 mL of sterile distilled water. Sufficient inoculum was added until the turbidity was equivalent to 0.5 McFarland standards (1.5 $x10^5$ cells/mL). One milliliter of the suspension was then added into 15.0 mL of sterilized molten Sabouraud dextrose agar aseptically. The mixtures were mixed well by swirling the plates left and right and then they were left on the bench to solidify. The commercial antibiotic disc GF A (Whatman) with 6.0 mm diameter was used to screen the anticandidal activity. Each of the sterile discs was then impregnated with 20 µL of the extracts, which corresponding to 100.0 mg/mL of extract. Ketoconazole at the concentration of 30 µg/mL was used as a positive control. On the other hand, 100% methanol was used as a negative control. All the impregnated disks were air dried before placing them on the agar surface. The plates were incubated at 37°C for 24 hours and the anticandidal activity was determined by measuring the diameter of the growth inhibition zones formed around the disc.

(iv) Determination of minimum inhibitory concentrations

The determination of MIC was applied by the broth macrodilution assay. A range of extract concentrations (0.78 to 100.00 mg/mL) was prepared in yeast extract-peptone dextrose (YPD) broth medium in flasks, and Tween 80 (a surfactant) was included at a final concentration of about 0.001% (v/v) to enhance extract solubility. A 16 hour old yeast cultures were diluted using sterile distilled water with reference to 0.5 McFarland standards to achieve inoculums size of $1.5x10^5$ cells/mL. Subsequently, each flask was inoculated with 20 µL/mL of yeast suspensions, homogenized and incubated at 37ºC in an orbital shaking incubator (100 rpm) for 24-48 hours. Flasks containing Tween 80 without the plant extract was taken as control. The lowest dilution of the extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism was recorded as the MIC value of the extract. Each test was performed in triplicate and repeated twice. A control experiment was run in parallel to study the impact of the solvent itself (without plant component) on growth of the test bacteria.

(v) Time-kill study of Candida albicans in the presence of methanolic extract of Wedelia chinensis leaves

C. albicans strain 1 was prepared as described previously and was harvested by centrifugation, washed twice with sterile distilled water and resuspended in sterile distilled water. The suspension was adjusted using the McFarland standard. The extract was added in to 25 mL of yeast extract-peptone dextrose broth in a 50 mL Erlenmeyer flask to achieve concentrations of 0 (control), 1.56 (1/2MIC), 3.13 (MIC) and 6.25 (2MIC) mg/mL after addition of the inoculum. The experiments were conducted in triplicate and all the flasks were incubated in a shaking (Infors HT Ecotron) incubator at 37°C with agitation at 100 rpm. One milliliter of the mixture within each flask was withdrawn at every 4 hourly intervals starting from 0 hour until 48 hours of cultivation and the cell growth was monitored by measuring optical density at 600 nm.

(vi) SEM and TEM observations

SEM observations were performed on *C. albicans* strain 1 cell suspension at a concentration of $1x10^6$ cells/mL was inoculated on a Sabouraud dextrose agar plate and incubated at 37ºC for 6 hours. Two milliliter of the extract at a concentration of 3.13 mg/mL was then dropped on to the inoculated agar and was further incubated for another 36 hours at the same incubation temperature. A methanol treated culture was taken as a control. A small block of *C. albicans* strain1 containing agar was cut and withdrawn from the inoculated plates at 0, 12, 24 and 36 hours of extract treatment. The agar blocks were fixed for scanning electron microscopy (FESEM LEO Supra 50VP, Carl Zeiss, Germany) works [27]. The remaining portion was used for transmission electron microscopy (Philips CM12, Eindhoven, Netherlands) works [28].

(vii) Statistical Analysis

The data obtained were analyzed by Student *t*-test for comparing the extract on the several strains of *C. albicans* against control, using SPSS Version 12.0. Statistical significance was assumed at the 0.05 levels (*p<0.05).

RESULTS

1. Anticandidal activity and minimum inhibitory concentration values

Anticandidal activity of the methanolic extract of *W. chinensis* leaves are shown in Table 1. The extract showed anticandidal activity with the diameters of inhibition zones ranging from 18.00±0.10 to 20.00±0.17mm. There were significant differences (*p<0.05) in the anticandidal activities of the extract. Subsequent experiments were conducted to determine the MIC values of the extract against the *C. albicans* strains. The minimum inhibitory concentration values ranged between 3.13 to 6.25 mg/mL. The results obtained appeared to confirm the anticandidal potential of the extract investigated. However, further investigation was concentrated on *C. albicans* 1 since it's showed the lowest MIC value in this study with only 3.13 mg/mL.

Table 1

Anticandidal activities of the methanolic extract of W. chinensis leaves against several pathogenic strains of C. albicans based on their zones of inhibition and minimum inhibitory concentrations.

***C. albicans* strains**	**Zones of Inhibition (mm)**		**MIC of the extract (mg/mL)**
	Crude extract (100 mg/mL)	Ketoconazole (30 µg/mL)	
Candida albicans strain 1	20.00±0.17	24.00±0.20	3.12
Candida albicans strain 2	18.00±0.10	22.00±0.15	6.25
Candida albicans strain 3	19.00±0.15	23.00±0.18	6.25
Candida albicans strain 4	19.00±0.17	24.00±0.16	6.25

Note: C. albicans 1- isolated from urinary tract infection, C. albicans 2- isolated from vaginitis, C. albicans 3- isolated from yeast onychomycosis, C. albicans 4- isolated from skin infection

2. Time-kill study

Time-kill study was conducted over a period of 48 hours with the aim to assess the anticandidal activity with ½ MIC (3.13 mg/mL), MIC (6.25 mg/mL) and 2MIC (12.5 mg/mL) values over time and the results are shown in Figure 1. At ½ MIC value, there were a drastic droped in OD after 12 hours of cultivation, which leads to the stationary phase of yeast growth compared to control (untreated cell). However, at MIC and 2MIC values, the extract eradicated the cell numbers. The results showed the potency of the extract as an anticandidal agent against *C. albicans*.

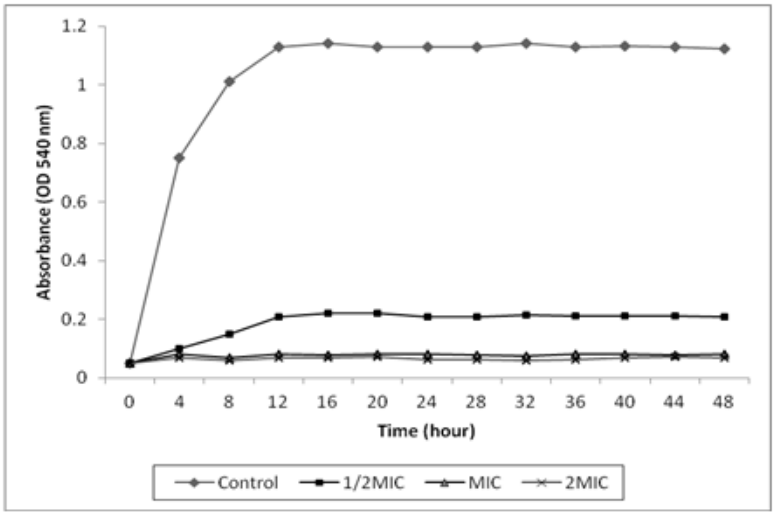

Figure 1
Growth profiles of C. albicans strain 1after treated with 1/2MIC (1.56 mg/ml), MIC (3.13 mg/ml) and 2MIC (6.25 mg/ml) of the methanolic extract of W. chinenisi leave.

3. SEM and TEM observations

A clearer view on the effect of the *W. chinensis* extract against the *C. albicans* cells are shown in Figure 2. Figure 2A shows many oval and smooth cells in appearance and some at a budding stage. After 12 hours of exposure (Fig 2B), there was some effect of the extract observed on the cells compared to the control cells. Figure 2C shows the 24 hours treated cells with a distinct cell formation with cavitation and shrunken cells. Finally, after 36 hours of exposure to the extract (Fig 2D), completely collapsed and cavitated cells were observed. At this stage the damage of the cells was beyond repair and the cells lost their metabolic functions completely.

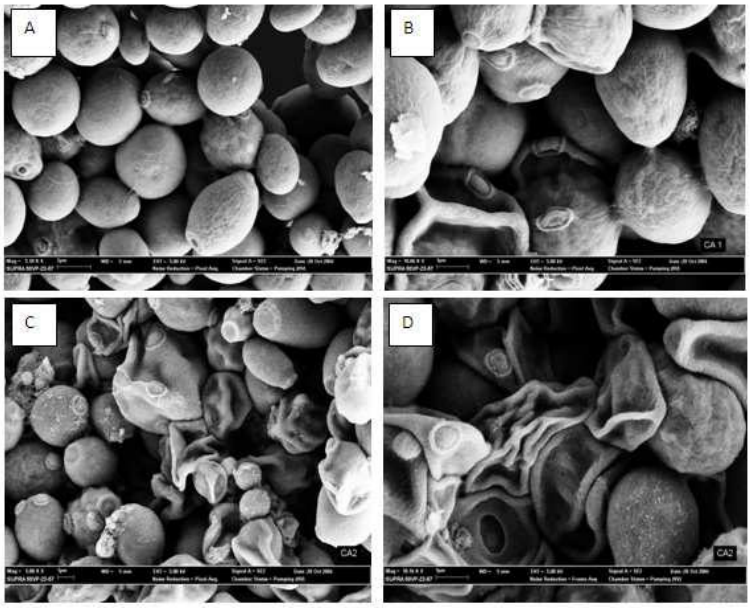

Figure 2
SEM micrographs of the untreated and methanolic extract W. chinensis leaves treated C. albicans strain 1 cells. (A) Untreated cells, (B) 12 hours, (C) 24 hours and (D) 36 hours of exposure.

Observation on SEM micrographs suggested the cells had undergone distinct morphological and cytological alterations. Further evidences of these changes can be clearly observed from the TEM micrographs which reaffirm some form of disorganization of the yeast cells and also destruction of its cytoplasm and organelles. Figure 3A shows a typical structure of *C. albicans* cells with nucleolus and organelles. The cytoplasm contains element of cell membrane system and enveloped by a typical cell wall of yeast cells. Figure 3B shows a 12 hour of exposure to the extract, cells were dense with the vesicles, membrane cell and cell wall disposition and altered within the cells. After 24 hours of exposure (Fig 3C), the cells exhibited notable alterations in the cell membrane and cell wall. The cytoplasmic volume decreased and there was deformed or stunted budding formation with disorganization within the cell cytoplasm. Figure 3D shows the effect of a prolonged exposure to the extract (36 hours), where the cells undergoing severe disorganization within the cells which led to collapsed cells and lysed.

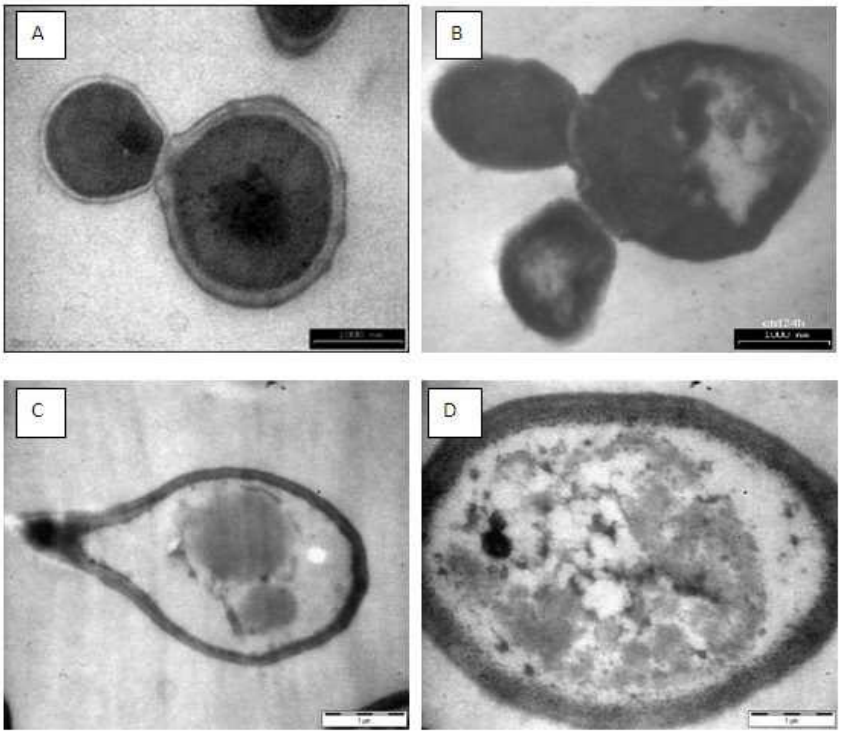

Figure 3
TEM micrographs of the untreated and methanolic extract W. chinensis leaves treated C. albicans strain 1 cells. (A) Untreated cells, (B) 12 hours, (C) 24 hours and (D) 36 hours of exposure.

DISCUSSION

Many antifungal agents are available to treat superficial and systemic mycosis. The emergence of drug-resistant strains and dose-limiting toxic effects has impeded antifungal treatment. Moreover, immune-compromised and hospitalized patients are more susceptible to severe fungal infections [29]. Many investigators have searched for new compounds with some antifungal action in natural products. Some plant extracts have been shown to possess activity against several pathogens and may be a good source of new bioactive agents. Many plants have been screened for activity against a wide array of diseases on the basis of ethnopharmacological data. In this study, methanolic extract of *W. chinensis* leaves was selected to be

investigated for its anticandidal activity, since the plant is used to treat various ailments and diseases [20], in which it is suspected to comprise of bioactive compounds that exert anticandidal activity. The results of our study showed the MIC values of methanolic extract of *W. chinensis* leaves were between 3.13 to 6.25 mg/mL which is considered higher than the MIC values for bacteria [30, 31]. Usually higher concentrations of extract are needed to inhibit the growth of yeast including *Candida* spp. Prabhakar et al. [32] reported that the ethanolic extracts of *Syzygium jambolanum* and *Cassia siamea* exhibited anticandidial activity at 100 mg/ml whereas *Odina wodier* was at 500 mg/ml. This could be due to yeasts are eukaryotic cells and their cell wall contains chitin, which totally different from cell wall component of bacteria. The MIC results had quantitatively demonstrated the anticandidal activity of the extract towards the susceptible cells. The succession of anticandidal activity of any plant extract depends on the ability of the antimicrobial agent to penetrate the cell wall. There are specific interactions between the bioactive compounds with the cell wall compartments [33]. This interaction with the constituents' target site will either aid or hinder the penetration of the bioactive compounds into the cells Morphogenesis which is the transition of unicellular yeast cells to the filamentous form (pseudohyphae), is an attribute of *Candida* species such as *C. albicans*. The presence of the filamentous form and budding is associated with virulence and pathogenicity, but both forms may be involved in the development and progress of disease. Several antifungal drugs and plant extracts can inhibit germ-tube formation and budding of yeast cells [34]. The methanolic extract of *W. chinensis* leaves at the concentration between 3.13 to 6.25 mg/mL inhibited morphogenesis of the yeast cells. These morphological changes can be observed from SEM and TEM micrographs. It was suggested that the effects of the extract were exerted on the outer membrane of the cell wall which then altered the cell membrane or plasma lemma structure and also the permeability of the cells. At the early stage (12 hour) the treated cells exerts a slight difference in their morphologies compared to control cells (untreated). However, the cells started to show more aberrant morphology over time. It can be summarized that the lethal action of the treated cells started with the shrinkage of the cells and then followed by the accelerated pores or cavity formations and unusual cell morphogenesis. It might have been due to the changes in the membrane cell structure resulted from the breakage of the hydrogen bonds that functions in keeping the rigidity of the membrane [35]. The same characteristics were observed in other antimicrobial studies of plant extract against pathogenic bacteria [30, 36]. Hyde et al. [37] suggested that the morphological changes of the antibiotic-treated bacteria occur when the antimicrobial agent attacked the cell membrane. The bioactive compound of the methanolic extract of *W. chinensis* leaves was locked on the cell surface structure and then permeabilized the cell membranes. Any disruption in cell wall integrity will have a great influence in the cell growth. There will be the loss of membrane potential, failure in cellular uptake of ethidium bromide and leakage of potassium ions and ATP [37]. This prediction was coincided well with the findings of Sasidharan et al. [38] who reported the methanolic extract of macroalgae *Gracilaria changii* exerted its inhibitory effect on the cell wall of the bacterial cells which led to the complete damage of the cells. Yogalatha et al. [39] also found the same results on the anticandidal activity of the methanolic extract of *Vernonia cinerea.* Various studies were reported to investigate the mechanism of actions involved in microbial killing process. Among them are the interactions of antimicrobial compound with the cell membrane [33]. As shown by the SEM micrographs where the cells became crumpled and exhibited formation of cavity. These damages may indicate the lost of cellular materials and organelles from the cell cytoplasm [40]. These unstable and altered cells were observed to be completely collapsed. Black [41] stated that a cell with non- rigid, non-sturdy, and abnormal usually tends to burse when expose to the low osmotic pressure. The interaction between the active compounds and the component in the cell wall of the yeast cells

also could lead to cell leakage. These conditions can be clearly observed in TEM micrographs of the extract treated cells compared to control, where the cell wall showed loss of integrity and low electro-density (Fig 3). In consequence, it induces modification of cell membrane or plasmalemma permeability and changes in cytoplasmic content. The results obtained from this study revealed that a severe modification occurs as the time of cells exposed to the extract prolonged. The exposure of yeast cells to sub-inhibitory concentration (1/2 MIC or 1.56 mg/mL) could change chalcone synthase (CSH) which was correlated with structural changes in the yeast cell wall [42]. There were reports on the uptake of antimicrobial compounds presence in the plant extract which affect greatly on the content in the cell wall of microorganisms [43]. The antimicrobial properties exhibited by the extracts may be associated with the presence of tannins, saponins, cardiac glycosides and alkaloids found in the methanolic extract of *W. chinensis*. A large number of flavonoids have been reported to possess antimicrobial properties [20, 24, 44, 45]. It is known that due to the presence of several phytochemical constituents like alkaloids, flavonoids, glycosides, saponins, tannins, steroids, terpenoids, phenolic compounds and so on, the plant extracts generally show antimicrobial properties [46, 47, 48, 49, 50]. The medicinal plants are known to provide a rich source of botanical antibiosis and many of them have been used because of their medicinal values in treating various ailments in human. Therefore, such plants should be investigated to better understand their properties, safety and efficacy. Furthermore, in most of the indigenous system of medicines, medicinal plants are used in their crude form, since there are many reports stated that the active substance of medicinal plants are unstable in nature when fractionated and they work well synergistically.

CONCLUSION

The present study conclusively demonstrates the anticandidal potential of the methanolic extract of *W. chinensis* leaves. The extract was proven to be fungistatic at lower concentration and fungicidal at higher concentration. In addition, phytochemical studies will be necessary to isolate the active constituents and evaluate the antiyeast activity against a wide range of yeast population.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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