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Research Article

Antiradical and Cytotoxic Activities of Varying Polarity Extracts of the Aerial Part of *Euphorbia hirta* L.

Shanmugapriya Perumal,[1] Roziahanim Mahmud,[1] Suthagar Pillai Piaru,[1] Wei Cai Lee,[1] and Surash Ramanathan[2]

[1] *School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[2] *Centre for Drug Research, Universiti Sains Malaysia, 11800 Penang, Malaysia*

Correspondence should be addressed to Shanmugapriya Perumal; angelpriya30@hotmail.com

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Euphorbia hirta is a well-known ethnomedicinal plant with diverse biological activities. The aim of the present study is to investigate the antiradical activities of various solvent extracts of the aerial part of *E. hirta* as well as to determine the possible cytotoxicity of these extracts. The aerial part of *E. hirta* was extracted with different solvent systems in order to increase polarity. The solvents used were hexane, dichloromethane (DCM), ethyl acetate (EA), ethanol (EtOH), and methanol (MeOH). The contents of total phenols and total flavonoids were analyzed by UV spectrophotometry, whereas the potential free radical-scavenging activities of the extracts were evaluated using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), the β-carotene-linoleic acid bleaching system, and reducing power. The EtOH extract exhibited the highest total phenolic content (237.9 ± 2.26 mg GAE/g), and DCM extract scored the highest total flavonoid content (121 ± 0.15 mg CE/g). The MeOH extract showed a potent free radical-scavenging activity as evidenced by low EC_{50} at 42.81 μg/mL. Interestingly, the EtOH extract demonstrated the highest reducing power activity with EC_{50} value of 6.18 μg/mL. In β-carotene-linoleic acid assay, oxidation of linoleic acid was effectively inhibited by DCM extract with 96.15 ± 0.78%. All the extracts showed no cytotoxic activity against Vero cells.

1. Introduction

The study of antioxidant from ethnomedicinal plants is producing a medical revolution due to their health-promoting benefits [1]. The increased discoveries in herbs and spices as sources of natural antioxidants have initiated researchers to look for natural antioxidants with low cytotoxicity [2]. In Malaysia, more than one hundred plant species are reported to have medicinal properties and amongst them is *Euphorbia hirta* with traditional claims in treating various diseases.

E. hirta L. belongs to the family Euphorbiaceae. It is an annual hairy plant that comprises about 300 genera and 5000 species, distributed in the temperate, subtropical, and tropical regions of the world. It is known by its Malay traditional names as "ara tanah" and "gelang susu". *E. hirta* is recognized by many vernacular names such as "pill bearing spurge," "cats hair," "asthma weed," "basri dudhi," "malnommee," and "fei yang cao". *E. hirta* exhibits interesting pharmacological activities and is much used in ethnomedicine in all tropical countries of Africa, Asia, America, and Australia. The plant is well known for the traditional claims in treating gastrointestinal and respiratory disorders such as ulcer, amoebic dysentery, diarrhoea, bronchitis, asthma, and hay fever. It is also used traditionally in the Philippines as a curative for dengue fever.

E. hirta is well acknowledged for its pharmacological activities such as antioxidant [3], antibacterial [4, 5], antifungal [6], diuretic [7], anthelmintic [8], antihypertensive [7], anxiolytic [9], antidiarrhoeal [10, 11], antimalarial [12], anti-inflammatory [13], and anticancer [3]. The current study was performed to investigate the various solvent polarity extracts of *E. hirta* as a potential antioxidant agent. To authenticate the safe use of these extracts as natural antioxidants for therapeutic application, a cytotoxicity assessment was executed. The findings from this study will help to promote the better human health.

2. Materials and Methods

2.1. Plant Collection and Authentication. The fresh aerial parts of *E. hirta* (Euphorbiaceae) were collected from an organic vegetable garden in Sungai Ara, Penang, during the period from January to March 2012. The plant was authenticated at the School of Biological Sciences by the institution botanist Mr. Shunmugam. A voucher specimen (no. 11254) is deposited at the Herbarium Unit of the Universiti Sains Malaysia.

2.2. Extraction of Plant Material. The aerial parts of *E. hirta* were air dried under sun shade for 5 days. The dried aerial parts were cut and grounded to mesh size no. 40 and macerated in different solvent systems in order to increase polarity. The solvents used were hexane, dichloromethane (DCM), ethyl acetate (EA), ethanol (EtOH), and methanol (MeOH) by ratio of 10 g of ground plant material into 100 mL of extracting solvent. The extraction was done for 3 days under occasional shaking, and the process was repeated three times at the room temperature. The extracts obtained were filtered and concentrated to dryness with a rotary evaporator (Rotavapor R-200, Buchi, Switzerland) under reduced pressure. The extracts obtained were eventually freeze-dried (FreeZone, MO, USA) to remove any residual water. The yield of all the extracts was determined, and the dried extracts were kept at 4°C until use.

2.3. Determination of Antioxidant Activity

2.3.1. Sample Preparation. All the extracts were redissolved in methanol at a concentration 1 mg/mL for determination of antioxidant contents.

2.3.2. Total Phenolic Content. The phenolic content in the extracts was determined using colorimetric assay [14]. Fifty microliters of extract samples were mixed with 1 mL of distilled H_2O in test tubes followed by addition of 0.5 mL of the Folin and Ciocalteu phenol reagent to the mixture. After 3 min, 2.5 mL 20% (w/v) Na_2CO_3 solution was added and the volume was filled up to 10 mL with distilled H_2O. The reaction was kept in the dark for 90 min incubation at ambient temperature after which the absorbance was measured at 735 nm against blank. The same procedure was repeated for standard gallic acid solutions, and total phenolic content was calculated using a calibration curve of gallic acid (0.05–1.0 mg/mL; $y = 0.0012x - 0.0141$; $r^2 = 0.9985$; y is the absorbance; x is the solution concentration). The experiment was carried out in triplicate, and the results were expressed as mg of gallic acid equivalents (GAE)/g of extract.

2.3.3. Total Flavonoid Content. Flavonoid contents were determined according to the method described by Zhishen et al. [15]. A volume of 250 μL of each extract was mixed with 1.25 mL of sterilized distilled H_2O and 75 μL of 5% $NaNO_2$ solution. A volume of 150 μL of 10% $AlCl_3 \cdot H_2O$ solution was added after 6 min incubation. Five hundred microlitres of 1 M NaOH solution were added after 5 min and then the total volume was made up to 2.5 mL with dd H_2O. Following thorough mixing of the solution, the absorbance was read against blank at 510 nm. The total flavonoid content was calculated using a standard curve of (+) catechin (0.06–1 mg/mL; $y = 2.8398x$; $r^2 = 0.9999$; y is the absorbance; x is the solution concentration). The results were expressed as mg catechin equivalents (CE)/g extract.

2.3.4. DPPH Free Radical-Scavenging Assay. The free radical-scavenging activities of the extracts were determined as per the method of Shimada et al. [16]. The concentrations of the tested samples ranged from 6.25 to 100 μg/mL. A volume of 50 μL of 1 mM DPPH (Sigma-Aldrich) in absolute methanol was added to 200 μL of each extract solution into the wells of 96-well microtitre plate. The solutions were mixed well, and the plate was incubated at room temperature for 30 min in the dark. The absorbance was measured against blank at 517 nm. Absolute methanol was used to zero the spectrophotometer, DPPH solution was used as blank sample, and BHT was used as a standard for comparison. The DPPH solution was freshly prepared and stored in a flask covered with aluminum foil and kept in the dark at 4°C between measurements. All experiments were carried out in triplicate. The radical-scavenging activities of the tested samples, expressed as percentage inhibition of DPPH, were calculated according to the following formula: $\text{IC}(\%) = [(A_0 - A_t)/A_0] \times 100$, where A_0 and A_t are the absorbance values of the blank sample and the test sample, at particular times, respectively [17]. Percentage of inhibition after 30 min was plotted against concentration, and the equation for the line was used to obtain the EC_{50} value. A lower EC_{50} value indicates greater antioxidant activity.

2.3.5. Estimation of Reducing Power (RP). The reducing power was determined according to the method of Oyaizu [18]. The various solvent extracts (31.25–500 μg/mL in methanol) were mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 200 ×g for 10 min. A 100 μL supernatant obtained hereafter was immediately mixed with 100 μL of methanol and 25 μL of 0.1% of ferric chloride, and the absorbance was measured after 10 min at 700 nm against blank. A higher absorbance value indicates higher reducing power. EC_{50} value (μg/mL) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis of graph absorbance at 700 nm versus concentration. The assays were carried out in triplicate, and the results are expressed as mean values ± standard deviations. Ascorbic acid was used as standard for comparison.

2.3.6. β-Carotene-Linoleic Acid Bleaching Assay. In this assay, antioxidant activity of the extracts was determined by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid

Table 1: Extraction yield, total phenolic, and total flavonoid contents of *E. hirta* aerial extracts.

Sample	Extraction yield (%)	Content	
		Total phenols (mg GAE[a]/g extract)	Total flavonoids (mg CE[b]/g extract)
MeOH extract	12.5	213.4 ± 0.75	62.3 ± 1.01
EtOH extract	10.9	237.9 ± 1.26	67.4 ± 0.76
EA extract	9.2	82.67 ± 1.73	70.4 ± 1.12
DCM extract	5.3	65.9 ± 0.85	121 ± 0.15
Hexane extract	4.4	45.9 ± 1.11	60.8 ± 1.11

Data are presented as (mean ± SD, $n = 3$).
[a]GAE: gallic acid equivalents.
[b]CE: catechin equivalents.

oxidation as described by Tepe et al. [19] with some modifications. A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 mL of chloroform (HPLC grade), and 25 μL linoleic acid and 200 mg Tween 40 were added. The chloroform was evaporated using a vacuum evaporator, and 100 mL distilled water saturated with oxygen (30 min 100 mL/min) was added with vigorous shaking. A 2.5 mL of this reaction mixture was dispensed into test tubes, and 350 μL portions of the extracts were added, and the emulsion system was incubated in hot water bath at 50°C for 2 h. The absorbance of these solutions was measured against blank at 470 nm using a Shimadzu UV-120-01 spectrophotometer (Shimadzu, Kyoto, Japan). The antioxidant activity was calculated in terms of percentage inhibition relative to the control. The inhibition percentage (I%) of the samples was calculated using the following equation: $I\% = (A_{\beta\text{-carotene after 1 h assay}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$, where $A_{\beta\text{-carotene after 1 h assay}}$ is the absorbance of β-carotene after 1 h assay remaining in the samples and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance of β-carotene at the beginning of the experiments. All samples were assayed in triplicate. Antioxidative capacities of the extracts were compared with those of butylated hydroxyl toluene (BHT) and blank.

2.3.7. Cytotoxic Activity. The varying solvent polarity extracts of *E. hirta* aerial part were tested for *in vitro* cytotoxicity, using Vero cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described by Mosmann [20]. Vero cells (ATCC, CCL-81) are mammalian epithelial cell line established from the kidney of the normal African green monkey (*Cercopithecus aethiops*). Vero cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL), and the cells were cultured at 37°C in a humidified 5% CO_2 incubator. Briefly, after being harvested from culture flasks, the cells were seeded at 1×10^6 cells in each well of 96-well plate containing 100 μL of fresh growth medium per well, and cells were permitted to adhere for 24 h. The cells were then treated with 100 μL of culture medium containing the test extracts with various concentrations into each well, with 1% dimethyl sulfoxide (DMSO) served as a negative control, whereas the medium only functioned well as a blank. After 48 h of treatment, the media in the wells were aspirated and replaced by 100 μL of 1 : 9 solution of MTT reagent in PBS to culture media. The MTT reagent was prepared as 5 mg/mL in PBS and mixed well with the appropriate amount of the culture medium. Then, the plates were incubated for another at 37°C for 3 h. After incubation, the media were aspirated, and 100 μL of DMSO were added into each well in order to dissolve the formazan crystals formed. The optical density of each well was then read using a microplate reader (Tecan, Switzerland) at a test wavelength of 570 nm and 620 nm as the reference filter. All test concentrations were run in triplicate, and the experiments were repeated thrice. The percentage of inhibition towards various concentrations of each test sample was then calculated using the following:

$$\text{Percentage of inhibition} = \left[\frac{\left(A_{\text{sample}} - A_{\text{blank}}\right)}{\left(A_{\text{control}} - A_{\text{blank}}\right)}\right] \times 100, \quad (1)$$

where, A_{sample} is the absorbance of the treated cells at a certain concentration of the extract, A_{blank} is the absorbance of the blank, and A_{control} is the absorbance of the cells treated with 1% DMSO.

The half maximal inhibitory concentrations (IC_{50}) of each test sample were then determined by plotting the values of percentage of inhibition against their respective concentrations. Consequently, the logarithmic regression equations obtained were used to calculate the IC_{50}. The results were expressed as mean ± SEM ($n = 3$).

2.4. Statistical Analysis. Statistical analysis was carried out by utilizing SPSS software version 16.0 (SPSS, Chicago, IL, USA). All experimental values shown are of mean ± SEM.

3. Results

The yield for each solvent extracts of *E. hirta* aerial part is shown in Table 1. The percentage of extraction yield declines as the solvent polarity decreases. The total phenolic content of various solvent extracts *E. hirta* aerial part was reported as gallic acid equivalent concentration (mg/mL). The highest contents of total phenol were found in the EtOH extract (237.9 mg GAE/g) followed by MeOH extract (213.4 mg GAE/g) (Table 1). In contrast, these two extracts possessed lower contents of total flavonoid which were (67.4 mg CE/g) and (62.3 mg CE/g), respectively. DCM extract exhibited the highest content of total flavonoids which was 121 mg CE/g. The hexane extract showed the lowest antioxidant contents (45.9 mg GAE/g for total phenols and 60.8 mg CE/g for total phenols) compared to all other extracts.

Table 2 illustrates the results for all the antioxidant tests performed in this study. In the DPPH test, assessed samples were able to reduce the stable violet DPPH radical to the yellow DPPH-H, reaching 50% of reduction with EC_{50} values ranging from 42.8 to 237.52 μg/mL. The scavenging effect of *E. hirta* aerial extracts and standard on the DPPH radical decreased in the order of BHT > MeOH extract > EtOH extract > hexane extract > DCM extract > EA extract. The free

Table 2: Antioxidant activities of *E. hirta* aerial extracts.

Sample linoleic	EC_{50} [a] of DPPH Free radical-scavenging activity (μg sample/mL)	EC_{50} [b] of reducing power (μg sample/mL)	Inhibition % of β-carotene-linoleic acid bleaching assay
MeOH extract	42.8 ± 0.34	44.3 ± 2.83	90.01 ± 0.97
EtOH extract	50.75 ± 0.19	6.18 ± 2.52	94.32 ± 0.36
EA extract	237.52 ± 0.76	141.14 ± 1.96	93.75 ± 0.42
DCM extract	197.83 ± 0.55	371.5 ± 3.15	96.15 ± 0.78
Hex extract	175.45 ± 0.15	661.75 ± 3.21	92.86 ± 0.53
Ascorbic acid	ND	36.1 ± 0.49	ND
BHT	18.5 ± 0.19	ND	93.2 ± 3.15

Data are presented as the means ± standard deviation (SD) of triplicate determinations.
[a]EC_{50} is the concentration required to inhibit radical formation by 50%.
[b]EC_{50} is the concentration for which the absorbance at 700 nm is 0.5.
ND: not determined.

radical-scavenging activity of MeOH extract was superior to all other extracts (EC_{50} = 42.8 ± 0.34 μg/mL) followed by EtOH extract (50.75 ± 0.19 μg/mL). The result of the reducing power of *E. hirta* aerial extracts from different solvents indicated that the EtOH extract had the highest reducing power with the EC_{50} value of 6.18 ± 2.52 μg/mL. The lowest reducing property was obtained from the hexane, DCM, and EA extracts with EC_{50} values of 661.75 ± 3.21, 371.5 ± 3.15, and 141.14 ± 1.96 μg/mL, respectively. In the β-carotene-linoleic acid bleaching system, oxidation of linoleic acid was effectively inhibited by the nonpolar extracts. DCM and EA extracts show 96.15% and 93.75%, respectively, that is higher than that of the synthetic antioxidant reagent BHT. On the other hand, MeOH extract (90%), EtOH extract (94.32%), and hexane extract (92.86%) displayed higher inhibition percentages which are very close to the BHT (93.2%).

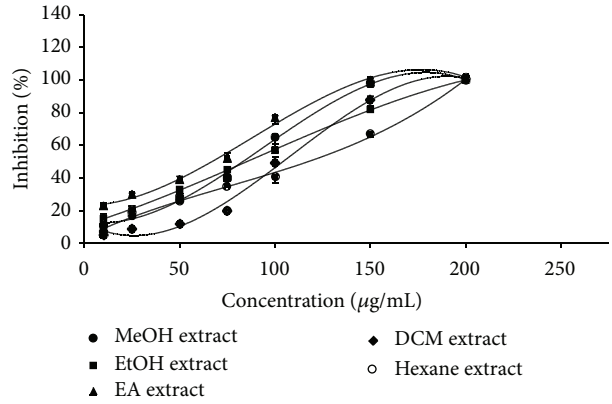

Figure 1: Cytotoxic activity of various solvent extracts of *E. hirta* aerial part on Vero cell line. The results were expressed as mean ± SD of triplicate determinations.

3.1. Cytotoxicity Effect of Plant Extracts. As shown in Figure 1, *E. hirta* extracts demonstrated no cytotoxic activity against Vero cells as all the IC_{50} values recorded are above the cytotoxic cut-off value of 20.0 μg/mL. The IC_{50} values for all the solvent extracts are as follows: MeOH extract (IC_{50}: 84.3 μg/mL), EtOH extract (IC_{50}: 95.6 μg/mL), EA extract (IC_{50}: 73.6 μg/mL), DCM extract (IC_{50}: 102.4 μg/mL), and hexane extract (IC_{50}: 137.8 μg/mL).

4. Discussion

There have been extensive reports on antioxidant properties of *E. hirta*. Mohammad et al. [21] had illustrated that different parts of *E. hirta* such as leaves, stems, flowers, and roots contained rich amount of phenols and flavonoids. However, the study on the antioxidant activity of the whole aerial part of *E. hirta* extracted with different solvent systems in order to increase polarity has not yet been reported elsewhere. Therefore, in this experiment the antioxidant activity of *E. hirta* aerial extracts was further studied by using solvents of different polarities for maceration such as methanol, ethanol, ethyl acetate, dichloromethane, and hexane, respectively.

Antioxidants are bioactive constituents that neutralize active components produced during metabolism process, such as free radicals which can harm the body [22]. Studies show that dietary consumption with high antioxidant content of phenolic compounds may decrease the occurrence rates of fatal diseases like cancer and cardiovascular [23, 24]. In their mode of action, the antioxidant potential of the aerial part of *E. hirta* can be mainly attributed to its radical-scavenging ability, reducing power, and singlet oxygen quenching ability. Free radicals are a major cause of the propagation stage of the oxidation process. The high potential for scavenging of free radicals could inhibit spreading of oxidation [25]. The effects of antioxidants *of E. hirta* aerial part extracts on the DPPH radical scavenging was determined by their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [26]. It is visualized as a discoloration of the reaction mixture at 517 nm. The MeOH and EtOH extracts of *E. hirta* exhibited stronger free radical-scavenging activities than nonpolar extracts. When compared to BHT, the MeOH extract is an almost effective radical scavenger.

The antiradical activity of plants is mainly contributed by the active compounds present in them. Many naturally occurring antioxidant compounds derived from plant sources have been identified as free radical or active oxygen scavengers [17]. The polarity of the solvent systems used in the extraction process affects the contents of phenolic and flavonoid components. Therefore, the levels of these components that existed in the various solvent extract varied significantly. In this study, the phenolics present abundantly in polar extracts compared to the nonpolar extracts. The data obtained from the total phenolic assay supports the key role of phenolic compounds in free radical scavenging. Phenolic compounds are well known to possess high antioxidant activities. Phenolics play an important role in the DPPH test and are responsible for the overall reactivity of the aerial extracts towards DPPH radical. In addition to phenolic constituents, the effectiveness of MeOH and EtOH extracts, *E. hirta* aerial part, as free radical scavenger is probably due to the high content of flavonoids, previously identified as a potential antioxidant. According to Agarawal [27], flavonoids are likely to be the most vital polyphenolic compounds that possess a broad spectrum of chemical and biological activities including radical-scavenging properties. The high potential of polyphenols to scavenge free radicals may be due to the presence of many phenolic hydroxyl groups. Thus, the presence of hydroxyl groups in the phenolic compounds makes the compound oxidize very easily.

The capability of certain antioxidants is often associated with their reducing power [28]. The reducing power of a compound depends on its ability in donating electrons and was observed to be concentration dependent. Generally, the extracts show significant reducing power reflecting the reductive ability, which was measured by $Fe^{3+} \leftrightarrow Fe^{2+}$ transformation in presence of test samples [29]. In the present study, the reducing power activity of EtOH extract was almost six times much higher than that of ascorbic acid. The MeOH extract showed comparable activity to that positive control (ascorbic acid) with EC_{50} value of $44.3 \pm 2.83\,\mu$g/mL. Therefore, the EtOH extract of *E. hirta* aerial part possessed the potent antioxidant compounds which are excellent electron donors that could terminate oxidation chain reactions by reducing the oxidized intermediates into the stable form. The EtOH extract also scored the highest phenolics content (Table 1) which may directly contribute to the strong reducing power. According to Kanatt et al. [30], phenolic compounds possess ideal structural properties to donate hydrogen atoms to free radicals.

β-Carotene is a colored molecule synthesized only in plants. β-Carotene bleaching is a free radical-mediated phenomenon, resulting in the formation of hydroperoxides from linoleic acid. In this model system, β-carotene undergoes rapid discoloration due to attack of free radicals formed upon abstraction of a hydrogen atom from the allylic methylene group of linoleic acid [29]. The oxidation reaction occurring in this model system can be spectrophotometrically monitored [31]. In the current study, although the phenolic and flavonoid contents are lower in the nonpolar extracts, the oxidation of linoleic acid may contributed by other secondary metabolites such as volatile oils, carotenoids, and vitamins. However, DCM extract displayed the highest total flavonoid contents of all and hence could be solely responsible for the bleaching activity. Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [32].

The cytotoxic effect of the varying solvent polarity extracts of *E. hirta* aerial part on Vero cell line was examined by MTT assay. The extracts displayed dose-dependent cytotoxic effects on Vero cells (Figure 1) with IC_{50} values above $20\,\mu$g/mL. The standard of cytotoxic or noncytotoxic was adapted from the guidelines set by the National Cancer Institute (NCI) which indicated that plant extracts or compounds with IC_{50} values less than $20\,\mu$g/mL are considered to be cytotoxic and noncytotoxic if otherwise [33, 34]. This finding is in good agreement with the traditional use of *E. hirta* plant extracts.

5. Conclusions

To conclude, the polarity of the extracting solvent plays a significant role in contributing antioxidant activities from *E. hirta* aerial part. The results from this study hence can be employed as a milestone in the further investigation particularly in isolating and characterizing the active principles that contribute to antioxidant activities. The extensive study will maximize the therapeutic effects of *E. hirta* as a natural antioxidant for use in pharmaceutical, food, and cosmetic industries.

Conflict of Interests

The authors report that they have no conflict of interests.

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