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A STUDY ON THE *IN-VITRO* ANTIFUNGAL, LARVICIDAL AND ANTIOXIDANT ACTIVITIES OF ROOT AND SHOOT OF *BIOPHYTUM SENSITIVUM* (LINN.) DC.

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Keywords:

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ABSTRACT: The present study assayed the antifungal, larvicidal and antioxidant properties of the aqueous and acetone extracts of root and shoot of *Biophytum sensitivum* (Linn.) DC. The aqueous extracts of both root and shoot had little activity against the three test fungi such as *A. niger*, *A. flavus* and *Penicillium*. The acetone shoot extract was also ineffective against these fungi. However, the acetone root extract exhibited magnificent antifungal activity against *A. flavus* (zone of growth inhibition of 33.5 mm); but moderately affected the growth of *Penicillium* (9.5 mm), in the disc diffusion assay. The acetone root extract had no activity on *A. flavus*. The aqueous extracts of root and shoot of *B. sensitivum* were also assayed for larvicidal activity on the the 3rd and 4th instar mosquito larval stages. The shoot extract exhibited potent larvicidal activity, with 100% mortality induced in the larvae, within 48 h of exposure with the extract. The root extract on the contrary, had negligible activity against the mosquito larvae, exhibiting only 30% larvicidal activity, even after 48 h of exposure with the extract. The antioxidant activity of the methanol extract of shoot and root of *B. sensitivum* was assayed by means of DPPH assay. The EC_{50} values obtained for root and shoot samples were 67 and 64 µg, respectively; the corresponding ascorbic acid equivalent antioxidant activities of these samples were calculated as 0.0746 and 0.078, respectively.

INTRODUCTION: *Biophytum sensitivum* (Linn.) DC is an important medicinal plant in the traditional Ayurveda system of medicine in India [1]. It is a member of the family Oxalidaceae and is distributed in tropical Asia, Africa, America and the Philippines. In India nine species of *Biophytum* are prominent; out of these three species *viz*. *Biophytum sensitivum* (Linn.), *Biophytum reinwardtii* (Edgew) and *Biophytum umbraculum* (Welw.) have been reported to possess ethno-medicinal properties [1].

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The vernacular names of the plant are Viparita Lajjalu, Jalapushpa, Krichhraha, Laghuvrishaka, Panktipatra and Peethapushpa in Sanscrit; Mukkutti, Nilaccurunki and Tintanali in Malayalam, Lakshmana and Lajjalu in Hindi, and Nilaccurunki and Teendanaazhi in Tamil [1, 2, 3, 4]. It is known for its interesting characteristic - inward curling of leaves in response to touch and hence the name 'Lajjalu'[1, 4].

B. sensitivum, commonly known as 'Life Plant' or 'Sensitive Plant', is a mesophytic under-shrub. This 'little tree plant' grows as an unbranched woody erect stem to a height of 2.5 to 25 cm. The leaves of the plant are abruptly pinnate, sensitive, 3.8-12.7 cm long and are made of 8-17 pairs of leaflets. The leaves grow on the top of the stem and are crowded into a rosette [2, 3, 5].

Leaflets are opposite, about 1 cm long; terminal pairs are the largest. Flowers are dimorphic and yellow, having a maximum of 8 mm in diameter and are crowded at the apices of the peduncles. The sepals are lanceolate, 7 mm long and parallely nerved. Corolla exceeds much larger than the sepals. Style is nearly glabrous. The flowers of this plant are significant for the people of Kerala, not only for its medicinal values but also for its cultural and traditional values. The flowers of this plant are valued as an inevitable part in Pookalams made during the festival of Onam [4]. The fruit is a capsule which is ellipsoid and apiculate. The seeds of this plant are ovoid, prominently ridged and transversely striated. *B. sensitivum* produces flowers and fruits from September to December [6].

B. sensitivum is rich in a number of phenolic and polyphenolic compounds, saponins, tannins, essential oil, polysaccharides and pectin [1, 4, 7, 8, 9, 10]. Mhaske and Gonjari have reported the presence of the two major bioactive principles, the bioflavonoids such as cupressoflavone and amentoflavone [10]. They have also reported the presence of tannins, saponins and some phenolic compounds in the leaves of *B. sensitivum*. Besides, the aerial part also contains three flavonoids (luteolin 7-methyl ether, isoorientin and 3'-methoxyluteolin 7-O-glucoside) and two acids (4-caffeoylquinic acid and 5-caffeoylquinic acid). The biochemical properties of the plant have also been attributed to the presence of 3′, 8″- biapigenin and proanthocyanidins [4]. The phytochemical analysis has indicated the presence of alkaloids, carbohydrates, flavonoids, proteins, cardiac glycosides, oils, phenolic compounds, terpenoids and tannins in the alcoholic extracts of *B. sensitivum* [11].

B. sensitivum is one of the auspicious herbs that constitute the group of 'Dasapushpam', an Ayurvedic formulation [12]. The whole plant (samoolam) of *B. sensitivum* is used in Ayurveda for the treatment of various diseases. The plant possess a multitude of therapeutic potentials that includes the analgesic, antipyretic, anti-inflammatory, immunomodulatory, antitumor, anti-diabetic, hypocholesteremic, antioxidant, anti-bacterial, antifungal, antihypertensive, chemo-protective, radioprotective, antifertility and wound healing properties [1, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22].

The plant is bitter, thermogenic, suppurative, expectorant, stimulant and tonic. It is useful in arthralgia, bursitis, cervical spondylosis, degenerative joint disease, degenerative neck disease, fibromyalgia, leg cramps, leg pains, osteoarthritis, rheumatoid arthritis, sprains, stiff neck, tendonitis, tennis elbow, urinary calculi, wounds, abscesses, asthma, phthisis, gonorrhoea, stomachalgia, insomnia and snake bite [5].

The dry powdered form of the whole plant is administered to cattle to prevent excessive salivation. In Philippines the powdered seeds are used as vulnerary, and along with butter, they are applied to abscesses to promote suppuration [1]. The powder, mixed with honey, is prescribed for abnormal growths and glandular swellings, and also in case of hypothyroidism. Ground leaves, when mixed with water, show diuretic effect and relieve thirst in yellow fever. In Philippines, the decoction of leaves of *B. sensitivum* is used as an expectorant and in Java it is used in the treatment of asthma. In Siddha system the ground leaves are administered along with butter milk for diarrhoea. The ground seeds are applied over wound and ulcer. The samoolam of this plant, mixed with honey, is used for curing cough and chest congestion. The leaf paste is applied over burns and contusions [4].

The ash of the plant, mixed with lime juice, is used for relief from stomach ache. Leaves and roots are styptic. The decoction of leaves is administered for diabetes, asthma and phthisis and also for treating varicocele. It is also used for treating heavy bleeding seen in women and hence the name 'Teendanaazhi'. It is a good medicinal herb to clean the uterus after delivery. The leaves of this plant, along with jaggery, is cooked and given to the delivered ladies to expel the lochia and remains from the uterus [23].

The potential of extracts of leaves of *B. sensitivum* as effective antibacterial agents has been reported against many species of bacteria such as *B. subtilis*, *B. thuringiensis*, *S. aureus*, *S. pneumonia*, *K. pneumonia*, *S. typhi*, *P. vulgaris*, *P. aeruginosa*, and *E. coli* [11, 24, 25]. *B. sensitivum* has also been reported to exhibit significant antifungal activity [25, 26, 27]. *B. sensitivum* has been found to have significant inhibitory effect on the growth of

mosquito larvae. A strong larvicidal activity for the methanolic extract of whole plant of *B. sensitivum* against the fourth instar larvae of *Culex quinquefasciatus* has been reported earlier [28]. There have also been reports on the larvicidal activity of leaves of *B. sensitivum* against *Aedes aegypti* [29]. In view of the phytochemical and microbicidal properties of the plant, the present study aimed at assaying the antifungal, larvicidal and antioxidant activities of the root and shoot extracts of the plant.

MATERIALS AND METHODS:

Collection of Sample: Fresh plants of *B. sensitivum* Linn. were collected from Kottayam District, Kerala, India. The plant was separated into two samples, the root sample and the shoot sample (constituting the aerial part that consisted of leaves, stem, flowers, fruits and seeds).

Fungal Strains Used: Fungal strains of the species *Aspergillus niger*, *Aspergillus flavus* and *Penicillium* were used in the study. These strains were collected from potato dextrose agar (PDA) plates after air exposure. The colonies were identified after observing the colony morphology and spore morphology. The fungi were isolated and purified and were maintained on PDA at 4 °C for further use.

Preparation of Extracts: The root and shoot samples of *B. sensitivum* were surface sterilized following the modified procedure of Aneja [30]. The samples were separately washed in running tap water for 10 min, followed by detergent wash in 10% Extran (Merck) for 10 min. The samples were washed again in distilled water for 10 min and were spread out in clean trays for oven drying. The root and shoot samples were oven dried at 60 °C, continuously, for 7 days. The dried samples were powdered using a clean grinder. The powder was stored in air tight containers, at room temperature, before extraction.

A fixed weight of 20 g of the powdered material was used for the continuous Soxhlet extraction for 8 h. The aqueous extracts were prepared at an extraction temperature of 100 °C and acetone extract at 60 °C. Each extract was concentrated by evaporation and made up to a final volume of 10 ml. The extracts were stored at room temperature, in sterile screw capped containers, until use.

Determination of Antifungal Activity: Sterile sensitivity discs of 6 mm diameter were prepared from Whatman no. 1 filter paper. The discs were sterilized by autoclaving at 121 °C, 15 lb pressure, for 15 min and stored at room temperature till use. The discs were soaked in the extracts for 2 min and allowed to dry. These dried discs were used for the antifungal assay.

Preparation of Fungal Spore Suspension: Fungal strains were cultured on PDA until the formation of fine confluent growth and fine spore formation was evident. 1 ml of sterile water was added to the plate and the spores were rubbed off from the surface of agar using a sterile brush. The spore suspension was collected in a sterile test tube and was used as the inoculum.

Disc Diffusion Assay: The aqueous and acetone extracts of root and shoot of *B. sensitivum* was tested for antifungal activity using disc diffusion method. Potato dextrose agar (PDA) was used for the antifungal assay of the extracts (HiMedia, Mumbai). Lawn cultures of the fungi were prepared on PDA plates using sterile cotton swabs charged with the fungal spores by dipping them in the spore suspension of the test fungi. Paper discs carrying the extracts were placed on the inoculated PDA plates (2 discs per plate). Discs carrying sterile distilled water and acetone were used as controls. The plates were incubated at room temperature for 48-72 h. The experiment was performed in duplicates.

Zone Analysis: After incubation the antifungal activity of the extracts against each fungus was assayed by measuring the diameter of zone of inhibition to the nearest mm. The results were recorded and compared.

Determination of Larvicidal Activity: The larvicidal activity was assayed using the crude aqueous extracts of root and shoot of *B. sensitivum* following the modified procedure of Dhanya and Benny [31]. The extracts were diluted in sterile water in the ratio 8:2 so as to make a final test volume of 10 ml. A negative control was set for the experiment using 10 ml of distilled water. The assay was carried out in duplicates. Mosquito larvae at the late III or early IV instar stage and measuring to a size of about 3-5 mm were collected

from stagnant muddy water. The larvae were exposed to 10 ml each of the crude extract in sterile Petri plates for 48 h. The larvae were considered dead if, within the experimental period of 24 h, they showed no signs of swimming movements even after touching with a glass rod. The data were analysed and the mortality rates, with respect to time, were recorded.

Antioxidant Assay:

Sample Preparation: 1 g dry weight each of the root and shoot samples of *B. sensitivum* were homogenized with 10 ml methanol for 1 min and were centrifuged at 10000 rpm for 15 min at 4 °C. The clear supernatant was transferred to sterile eppendorf tubes and measured immediately for total antioxidant activity using DPPH assay.

For this the root and shoot samples were made into different concentrations and 10 µl each of these diluted samples were analyzed so as to get the EC_{50} value (Effective Concentration) which is the concentration of antioxidant able to destroy 50% of the initial DPPH.

DPPH Reagent Preparation: The stock solution was prepared by dissolving 0.0025 g of DPPH in 10 ml methanol and covered with aluminium foil. The working standard was prepared by using 6 ml of the stock solution and making it up to 60 ml with methanol.

Evaluation of Free Radical Scavenging Activity by DPPH Method: The determination of radical scavenging activity of shoot and root of *B. sensitivum* was assayed using the DPPH assay following the modified method of Mensor *et al.* [32] Various concentrations of root and shoot extracts of *B. sensitivum* were taken in a series of test tubes and made up to 50 µl with methanol. Methanol was used as control. 2 ml of DPPH working standard was added to all test tubes including control.

The tubes were allowed to stand in dark, at room temperature, for 20 min. The reaction was carried out in triplicates. The decrease in absorbance was measured at 515 nm on a spectrophotometer. The scavenging activity of the samples corresponds with the intensity of quenching DPPH. Lower the absorbance of the reaction mixture, higher the free radical scavenging activity.

The capability of scavenging the DPPH radical was calculated by using the following formula.

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where A_0 is the absorbance of the control reaction and A_1 is the absorbance of test. All the tests were performed in triplicates and the average of the results were used for data analysis. The EC_{50} of the samples were compared with that of ascorbic acid as standard. The antioxidant capacity of ascorbic acid is equivalent to vitamin C equivalents per mg dry wt. Hence the ascorbic acid equivalent antioxidant capacity of the samples can be calculated as [EC_{50} vitamin C (mg/ml) / EC_{50} sample (g/ml)].

RESULTS AND DISCUSSION: *Biophytum sensitivum* (Linn.) DC, a perennial herb of Indian origin, is a rich source of secondary metabolites, often used for the treatment of chest complaints, convulsions, cramps and inflammatory tumors. *B. sensitivum* has been studied to a great extent for its antioxidant [13], immunomodulatory [14], antitumour [19], anti-inflammatory [8], antidiabetic [20], chemo-protective [15] and wound healing properties [16]. The research carried out so far has revealed the pharmacological potential of *B. sensitivum* and the *in vivo* studies has proved that most of these compounds are relatively safe. The present study tried to investigate the potential of root and shoot extracts of *B. sensitivum* as antifungal and larvicidal agents and also as antioxidants.

Antifungal Activity of Root and Shoot Extracts of *B. sensitivum*: The results of antifungal activity of root and shoot extracts of *B. sensitivum* has been represented in **Tables 1** and **2**, respectively. In the current study, the acetone root extract of *B. sensitivum* exhibited strong antifungal activity against *A. flavus* with a zone of growth inhibition of 33.5 mm **Table 1**. The extract was also inhibitory to the growth of *Penicillium* also, though in a weaker manner (9.5 mm). Unlike the acetone root extract, the acetone shoot extract failed to inhibit the growth of *A. flavus* or *Penicillium* **Table 2** in this study. Neither of the acetone extracts inhibited the growth of *A. niger*. The aqueous root and shoot extracts of the plant completely failed to inhibit the growth of *A. flavus, A. niger* or

Penicillium. Vijayan *et al*., [27] has concordantly reported the antifungal activity of leaves of *B. sensitivum* against *A. niger* and also against other fungi such as *A. fumigatus*, *C. neoformans* and against streptomyces like *Nocardia*. On the contrary, Narendran *et al*., [25] has previously demonstrated the inhibitory effect of aqueous extract of *B. sensitivum* on the growth of *A. niger*. Kala *et al*., [26] has reported the antifungal activity of leaf callus extract of *B. sensitivum* against other fungi *viz. Trichoderma viridae, Helminthosporium solani* and *Candida albicans*.

In this study the acetone root extract alone was effective as a fungicidal agent, particularly for *A. flavus*. Most of the studies on the antimicrobial activity of *B. sensitivum* (Linn.) DC has been focussed on the leaf and stem extracts or of the whole plant. This is perhaps the first study of *B. sensitivum* assaying the antifungal potential of the root extract of the plant. The study becomes significant as the root extract is exhibiting magnificent antifungal activity against *A. flavus*.

TABLE 1: ANTIFUNGAL ACTIVITY OF ROOT EXTRACTS OF *BIOPHYTUM SENSITIVUM* (LINN.) BY DISC DIFFUSION METHOD

Fungal Strains	Root extracts of *B. sensitivum* (Linn.) (Average Diameter of Zone of Inhibition of Growth in mm)	
	Aqueous	Acetone
A. niger	0	0
A. flavus	0	33.5
Penicillium sp.	0	9.5

TABLE 2: ANTIFUNGAL ACTIVITY OF SHOOT EXTRACTS OF *BIOPHYTUM SENSITIVUM* (LINN.) BY DISC DIFFUSION METHOD

Fungal Strains	Shoot extracts of *B. sensitivum* (Linn.) (Average Diameter of Zone of Inhibition of Growth in mm)	
	Aqueous	Acetone
A. niger	0	0
A. flavus	0	0
Penicillium sp.	0	0

Larvicidal Assay of Aqueous Root and Shoot Extracts of *B. sensitivum*: In the larvicidal assay the potential of aqueous extracts of root and shoot of *B. sensitivum* Linn. were assayed against the late III or early IV instar mosquito larvae. The crude aqueous extracts of root and shoot of *B. sensitivum* were used for the assay and the mortality rates were calculated after verifying the number of dead larvae during the test period that started form the moment of exposure to the respective extracts, to the final stage of the experiment at the end of 48 h. The mortality rates were calculated for the crude extracts (test samples T) and compared with the larvicidal activity of water as control (C).

Of the two extracts used against mosquito larvae, 50% larvicidal effect (LC_{50}) was exhibited by the shoot extract even within 1 h of treatment and 60% larvicidal effect after 3 h of treatment. The mortality rate was 90% after 24 and 30 h of exposure of the larvae to the aqueous shoot extract **Table 3**; **Fig. 1**. The mortality rate reached 100% within 30 to 48 h of exposure. The root extract exhibited only negligible activity in the larvicidal assay. The mortality rate was only 30% after 16 h of exposure of the larvae to the aqueous root extract of *B. sensitivum* **Table 3**; **Fig. 1**. The same was observed for the root extract even after 24, 30 and 48 h of exposure, which marked the end of the observation period.

TABLE 3: LARVICIDAL EFFECT OF AQUEOUS SHOOT AND ROOT EXTRACTS OF *B. SENSITIVUM* (LINN.)

Test sample	No. of dead larvae											
	0 h		3 h		5 h		24 h		30 h		48 h	
	T	C	T	C	T	C	T	C	T	C	T	C
Root	0	0	0	0	0	0	**3**	0	3	0	3	0
Shoot	0	0	6	0	6	0	9	0	9	0	10	0

T - Test sample, C - Control sample

This study suggests the use of aqueous shoot extract of *B. sensitivum* as an alternative for other larvicidal agents as it exhibits 50% mortality of the larvae even within 1 h of exposure. Shivakumar *et al*., [29] has demonstrated similar results while studying the larvicidal activity of leaves of *B. sensitivum* against *Aedes aegypti* where the leaf extract was found to interfere with the normal development and emergence of the larvae in a dose dependent manner.

The larvicidal activity of methanolic extract of whole plant of *B. sensitivum* against the fourth instar larvae of *Culex quinquefasciatus* has also been reported earlier [28]. The previous studies on the larvicidal activity of the plant were either using the whole plant or the leaves of the plant. This is the first study reporting the larvicidal activity of root extracts of *B. sensitivum*.

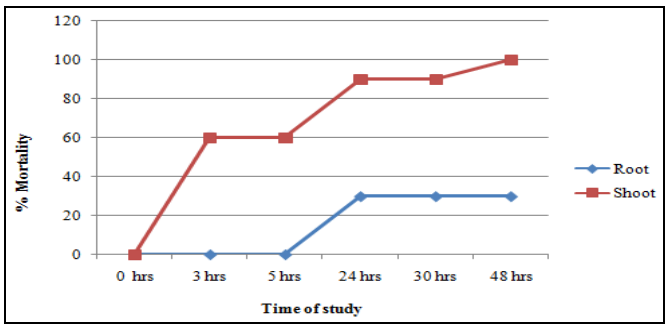

FIG. 1: PERCENTAGE MORTALITY OF MOSQUITO LARVAE ON EXPOSURE TO AQUEOUS ROOT AND SHOOT EXTRACTS OF *B. SENSITIVUM*

Antioxidant Activity of Shoot and Root Extracts of *B. sensitivum*: The antioxidant potential of *B. sensitivum* has been proved earlier by *in-vitro* as well as *in-vivo* studies [33]. The antioxidant activity of the methanolic extract of root and shoot of *B. sensitivum* was assayed in this study by evaluating the free radical scavenging activity of these extracts using DPPH assay. The dry weight of root extract was 0.098 g/ml and shoot extract was 0.084 g/ml. The samples were prepared in different concentrations to calculate the EC_{50} value. The EC_{50} valve of Vitamin C was calculated as 5 µg. In this study, the EC_{50} value obtained for root and shoot samples were 67 and 64 µg, respectively. The ascorbic acid equivalent antioxidant capacity for root and shoot samples were calculated as (5/67) 0.0746 and (5/64) 0.078, respectively **Fig. 2**.

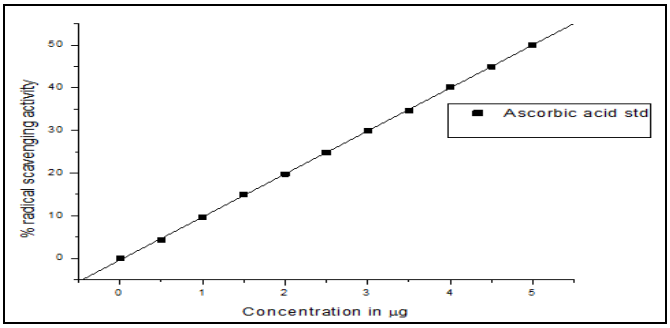

FIG. 2: RADICAL SCAVENGING ACTIVITY OF ASCORBIC ACID

B. sensitivum extract has been found to scavenge superoxide radicals, hydroxyl radicals and inhibit *in-vitro* lipid peroxidation at concentrations of 50, 95 and 20 µg/ml (IC_{50}) respectively [33]. Intra-peritoneal administration of *B. sensitivum* extract has inhibited superoxide generation in macrophages *in-vivo* in mice. The extract has also been reported to produce significant increase in catalase activity, glutathione, glutathione-S-transferase and glutathione reductase.

The levels of glutathione peroxidase has been found decreased after administration of *B. sensitivum* extract [33]. Kala *et al.*, [34] has also observed the *in-vitro* inhibition of activities of enzymes like α- glucosidase, 5- lipoxygenase, acetyl cholinesterase and tyrosinase in the pharma-cological investigations of *B. sensitivum* callus methanol extract. The low level of antioxidant activity of the callus extract has been attributed to the presence of lower concentration of phenolic compounds in the extract. This is the first study assaying the antioxidant activity of root and shoot of *B. sensitivum* separately and the results of this study proved that both the root and shoot extracts has good antioxidant activity, that too in similar range.

CONCLUSION: *Biophytum sensitivum* (Linn.) DC is a small flowering plant that contains a multitude of bioactive compounds which includes bioflavonoids such as amentoflavone and cupresso-flavone, alkaloids, carbohydrates, flavonoids, proteins, cardiac glycosides, oils, phenolic compounds, terpenoids and tannins [4, 7, 8, 9, 10, 11]. The current study is significant in the fact that here the root and shoot samples of the plant were assayed separately, unlike in other cases where the whole plant or leaves of the plant were employed for most of the assays.

Here the efficacy of root and shoot samples of *B. sensitivum* (Linn.) DC as potential antifungal and larvicidal agents and as effective antioxidants was investigated. The acetone root extract showed significant antifungal activity against *A. flavus* while the other extracts had little activity on the growth of the test fungi. The shoot extract was significantly larvicidal within 5 h of exposure. The root and shoot samples also exhibited good antioxidant activity in almost similar range. This is a preliminary study reporting the antifungal activity of the acetone extract of the plant. The phytochemicals responsible for this may be identified and also a quantitative assay will help in identifying the load of these compounds in the plant. The larvicidal activity of shoot extract of *B. sensitivum* could be exploited for the biological control of mosquitoes and mosquito-borne diseases, which will totally eradicate problems posed by synthetic insecticides such as insecticide resistance, pollution and toxic side effects on human beings.

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CONFLICT OF INTEREST: The authors declare no potential conflicts of interest.

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