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Research Article

In vitro propagation of threatened therapeutic tree *Buchanania lanzan* Spreng (Anacardiaceae)

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Abstract

Embryo rescue refers to an *in vitro* technology have been used for propagating plants under *in vitro* from poorly developed embryos by avoiding embryo abortion. The aim of the present study was to *in vitro* regeneration of a medicinally wild tree species *Buchanania lanzan* through embryo rescue method. Isolated immature poorly developed embryo dissected out from de-coded seeds collected from 15 years old trees were cultured an Woody Plant Medium (WPM) was used to test *in vitro* germination. 100% embryo germination were recorded when the embryos inoculated on the medium supplemented with 1.5 mg/L^{-1} 6-benzylaminopurine (BAP) and 50 mg/L^{-1} adenine sulphate (AS). Further, a week-old seedling derived explants viz embryo axis and cotyledonary node were selected to study *in vitro* clonal propagation. On medium augmented with 1.5 mg/L^{-1} 6-benzylaminopurine (BAP) showed maximum mean shoots numbers (4.2±1.80) with 4.0±0.98 cm shoot height were achieved in cotyledonary node. Compare to Kinetin (Kn) BAP was to be better in shooting response and multiple shoot induction. Healthy regenerated shoots was separated from multiple shoots cluster and transferred to a rooting medium containing half strength WP medium fortified with Indole-3-butyric acid (IBA). IBA at 0.5 mg/L^{-1} showed 90% rooting response and maximum mean roots (4.9±0.98) per shootlet was obtained. The rooted *in vitro* plantlets were transferred to polythene bags containing autoclaved humus, red soil and farmyard manure (1:1:1 ratio) for acclimation at 25 ± 2 °C. Later, these plantlets were gradually transferred to field condition. About 70% survival rate was recorded after three months period. The protocol developed in the present study was to restore the viable embryos from poorly developed immature embryos and rapid multiplication can help to *ex situ* conservation of this threatened tree *B. lanzan.*

Keywords: Woody plant medium, *In vitro* seedlings, 6-benzylaminopurine, Adenine sulphate, Indole-3-butyric acid

INTRODUCTION

The genus *Buchanania* is a medicinally important fruit bearing tropical dry deciduous forest trees in the world [1]. It was originated in the Indian sub-continent, and it is distributed in India, Burma, Nepal, Myanmar, Sri lanka and Malaysia [2]. Among the seven species, two species of the trees namely, *Buchanania lanzan* & *Buchanania axillaris* produce edible fruits species reported in India [3]. *Buchanania lanzan* commonly known as cuddapah almond belongs to the family Anacardiaceae. The plant leaves are 6-10 inches long, with oblong and obtuse in nature. Its flowers are whitish green, sessile and fruit is drupe type. It is green when at immature state and dark black at ripened stage. The phenology of the tree starts from January to March and fruits ripen in the month of April-June every year [4]. Traditional knowledge reveals that various parts of the tree such as roots, leaves, fruits, seeds and gums has higher medicinal properties such as anti-inflammatory, antioxidant, etc., was reported [5-7]. Bark or its leaf paste and *Diospyros melanoxylon* mixed with a glass of water is given to treat snakebite [8]. Ointment prepared from its kernel is applied to relieve itch and prickly heat. The gum derived from the bark is used for treating diarrhea and pains. The leaves are used for the treatment of wound and skin diseases [9, 10]. This tree species served as a significant source of livelihood for tribal populations.

Due to indiscriminate collection and over-exploitation for its medicinal use and high commercial market value this tree categorized under the threatened medicinal plant list. Conventional vegetative propagation was low due to erratic rooting behavior of stem cuttings and poor seed germination. A considerable reduction in wild populations of this tree in the forest areas has been recorded and facing a severe threat of extinction [11]. Hence, *ex-situ* conservation of this threatened medicinal tree is an impending need [3]. Embryo rescue method is the recovering of immature or mature zygotic embryo which normally abort and culturing on artificial nutrient media

under aseptic environment in order to develop vigor and viable plants through a successful ontogeny [12]. Earlier, an attempt was made to propagate this plant under *in vitro* studies with lower frequency was reported [1, 5]. Therefore, the present work was carried out to develop an improved *in vitro* regeneration protocol through embryo rescue method using immature embryo explants.

MATERIAL AND METHODS

Field survey and plant collection

The present study, attempted by frequent field work at a weekly intervals and assessed the flowering patterns, fruit set and seed development & its dispersal of this medicinal tree *B. lanzan*. This chosen study areas based on where we could locate the taxa in the wild habitat. The field study area was South Western Ghats (Sathuragiri Hills (1500 ft), Palani Hills (up to 2700 ft) and Eastern Ghats (Sirumalai Hills (up to 5, 200 ft), Alagar Hills (up to 1200ft.) Tamil Nadu, India. The field work was carried out from the period of January 2018 to June 2020, which was the normal season for every year for flowering and seed setting of this vulnerable tree. In the survey, we had studied, the biological difficulties faced of the above said characteristics of this ill-fated tree. The medicinal woody species were collected from study areas of the natural habitat during dry and rainy seasons (Fig.1a). The specimen was authenticated and deposited at Sri Ganesan Herbarium Centre (SGHC), Botany Department for future reference (Voucher No. SGHC 510). The plant was also established in the experimental garden, Department of Botany, The Madura College, Madurai, Tamil Nadu, India.

Surface sterilization and source of explants

The immature fruits of *B. lanzan* were collected from the savanna forest of selected study areas of Tamil Nadu, India were used as a source of explants. The seeds were manually dissected out from de-coated immature fruits were washed thoroughly in running tap water for 30 minutes, followed by treatment with a solution of 3% Tween 20 (w/v) for 15 minutes. Then the explants were treated with 70% (w/v) ethanol for a minute subsequently, they were surface sterilized with 0.5% (w/v) mercuric chloride for 15 minutes. Later, in order to remove adhesive chemical residues on the seeds surface were washed with sterile double distilled water for five to six times.

Culture medium and incubation

The immature embryos were dissected using sterile forceps and scalpel blade from surface disinfected seeds. The explants were inoculated on semi-solid Wood Plant (WPM) [13] basal medium was prepared following standard protocol. The basal medium contained sucrose 2% (w/v) and agar 0.8% (w/v). The pH of the medium was adjusted to 5.8 by using 1N NaOH/ 1N HCl after adding the plant growth regulators (PGRs). The media was steam sterilized in the autoclave at of 1.05 kg/cm^2 pressure and 121^0 C temperature for 15 minutes. The cultures were incubated at 25 ± 2 ^{0}C temperatures under 16 h photoperiod provided by white fluorescent tubes (55 µmol/m^2/sec.).

Embryo germination, *in vitro* shoot initiation and further multiplication

To study embryo germination the immature embryos were cultured on the medium supplemented with 2.0 mg/L^{-1} (BAP) and 50 mg/L^{-1} adenine sulphate (AS). The percentage of germination was recorded. For *in vitro* shoot initiation and multiplication, a week-old seedling derived explants viz embryo axis and cotyledonary node were cultured on basal medium without PGRs. The medium also supplemented with various concentrations (0.5 to 3.0 mg/L^{-1}) of 6-benzylaminopurine (BAP) and Kinetin (Kn) (0.1 to 1.5 mg/L^{-1}) were used for multiple shoot initiation. For further shoot multiplication and elongation subcultures were made at three weeks durations on the optimal concentrations of cytokinins and results were recorded. The frequency of explants produced shoots, number of shoots per explants and shoot length were also recorded after culture period.

In vitro root induction and plant acclimatization

Healthy well developed *in vitro* regenerated shoots of the tree were separated from multiple shoot clusters and transferred to rooting medium containing half strength of Woody Plant medium fortified with different concentration (0.1 to 2.0 mg/L^{-1}) of indole-3-butyric Acid (IBA), indole-3-acetic acid (IAA) and Naphthalene acetic acid (NAA) individually. Well-rooted plantlets were successfully transferred to black polythene bag containing autoclaved soil and covered with transparent aerated polyethylene bags to maintain optimal relative humidity (RH). After 20 days, the hardened plants were transferred to earthen pots containing mixture of humus, red soil and farmyard manure (1:1:1 ratio). The pots were watered periodically at regular intervals under controlled environmental conditions. The percentage of survival rate was recorded after 6 weeks after transplantation. Finally, fully acclimatized plants were progressively grown in the natural field condition.

Statistical analysis

The experimental set up is completely randomized design. The data were collected at the end of the culture period. Each treatment consisted of 20 replicates and all experiments were repeated at least three times. All data were subjected to significance was once determined by using calculating the Mean ± Standard Deviation in One-way ANOVA using SPSS statistical software package (Trial version: 16) was used.

RESULTS AND DISCUSSION

Field observation: Problems associated with the flowering and fruiting of *B. lanzan*

Based on our field survey, the following observations were attempted on the major bottlenecks of *B. lanzan* in its flowering and fruit development. Figure 1 showed various critical stages of flowering and fruit setting. The normal flowering season of the species was started from January to June every year. It was found that, there is an intermittent flowering occurs in the selected tree which is growing in a same geographical study area (Sathuragiri, Palani, Sirumalai & Alagar hills). The inflorescence bud formation and flowering were seen normal at earlier stage (Fig. 1b & c). After a couple of month periods (February), less numbers of flowers were pollinated by insect pollinators and fewer numbers of fruits are formed with poor embryo development at final stage (Fig. 1d). At the end of fourth mouth (March) the entire pyramidal inflorescence axis along with fruits turns in to burn and dry, as a result it was dead (Fig. 1e). However, those fruits are collected from other months were found to be less responsive, exhibited more disappearing and contamination with infestation of fungal mycelium. The seasonal effect of explants

for culture establishment has also been reported in *Celastrus paniculatus* [14]. The presence of hard seed coat is one of the shelling problems in decortications of nuts; it is miniature size ready to damage the embryo at the time of decortications. The seeds has a low storage stability is another bottle neck [15, 16]. We have noticed that, natural conducive humidity and massive temperatures triggers fungal invasion and enhances profuse mycelial growth makes damages further, so the fruit developmental changes of this threatened taxon and disturbed life cycle of this vulnerable tree species. Our findings clearly showed that, it could be major biotic factors which influences to dwindling in numbers in natural habitat of this tree species. Similar study was other plants species reported on [17] .

Figure 1: Difficulties in flowering and fruiting stages of *B. lanzan* (Spreng) (a) Natural habitat, (b & c) Inflorescence lower initiation and blooming, (d) Drying of pyramidal panicle after one month, (e) Fewer fruit formation after two months.

Embryo rescue and seedlings development

Embryo rescue techniques have been widely used for propagating plants under *in vitro* from in which failure of endosperm to properly develop causes embryo abortion [18]. During fruits development seeds with aborted embryo were common in most arbores species due to various biotic factors [19]. In the present study we have noticed that partially developed aborted embryo because of invasion of fungal spores and aggressive mycelia growth of this species. In order to overcome this problems embryo rescue techniques as a promising tool widely used for producing plants from woody plant species. In embryo rescue procedures, the woody plant medium nutrients serves as a substitute for the endosperm development, in that way allowing the embryo to restore and continue its further development.

The present study, from 3 weeks (Day after Pollination, DAP) old fruits of *B. lanzan* poorly developed zygotic embryos were carefully isolated using stereomicroscope and inoculated on WP medium. The immature embryos were cultured on medium incorporated various concentrations of cytokinins showed higher rate of embryo germination (Fig. 2 a). In this study, we have achieved 100% embryo germination at 2.0 mg/L^{-1} BAP with 50 mg/L^{-1} (AS) (data not shown). No embryo germination was observed in control medium without PGRs. The synergistic effect of BAP along with adenine sulphate is act as a most favorable nutrient substitute for embryo germination and shoot formation was reported in earlier studies [20-21]. Initially explants were expanded three fold from in its original size in a week culture period (Fig. 2 b). The shoot initiation was noted after three weeks cultures (Fig. 2 c). About 76% shooting response was noticed on medium with 1.5 mg/L^{-1} BAP and showed maximum number of shoots (5.8±1.58) and height (5.2±1.25) of the shootlets (Table 1). Kinetin supplemented medium produced significantly fewer shooting response. At kinetin 1.5 mg/L^{-1} produces a maximum number of shootlets (1.0±0.79) with 60% shooting response. BAP was found to better than Kinetin in shooting response and shoot multiplication. In the optimized hormonal concentrations of the medium enhances lateral buds proliferation was achieved. Similarly, the efficiency of BAP in axillary shoot bud induction has been well reported in *shorea robusta* [23].

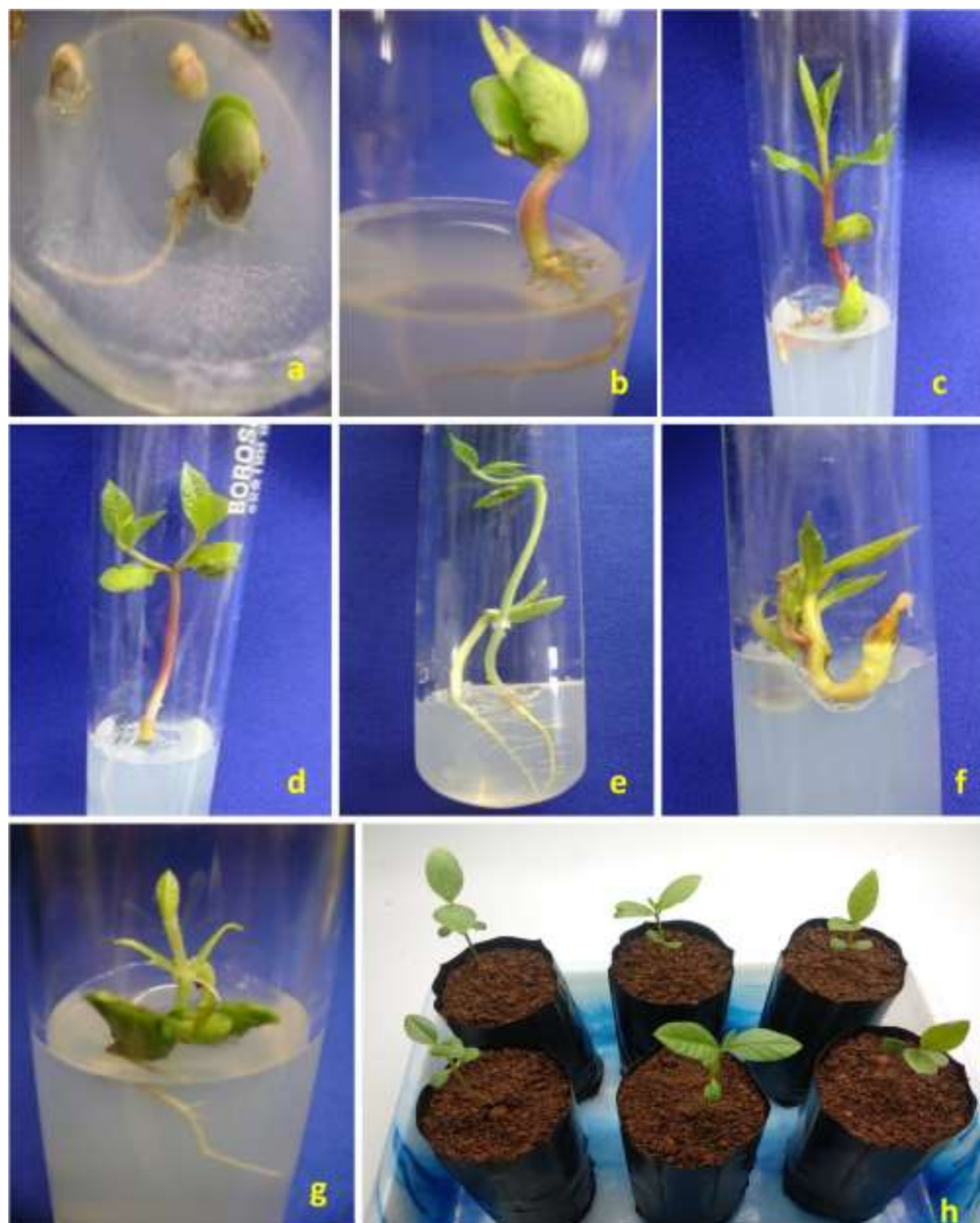

Figure 2: *In vitro* embryo rescue of *Buchanania lanzan* (spreng) (a) Embryo germination after 14 days, (b) Initiation stage of seedling development, (c,d & e) Further shoot development, elongation and rooting, (f) Shoot deveopment from embro axis, (g) Shoot formation from Cotyledonary nodal bud, (h) Seedlings derived whole plantlets from various explants acclimatized in polybags.

***In vitro* plant regeneration from seedling derived explants**

The achievement has been made in the *in vitro* propagation of many woody species, such as *Vitex negundo, Albizia lebbeck,* and *Tecomella undulata* [24]. Even though some tree species can be micropropagated explants derived from mature trees, many others can presently be propagated only from tissues of juvenile parts to avoid phenolic exudates [25]. In our study, embryo axis and cotyledonary nodes excised from seedlings were used as a primary source of explants for shoot development and plant regenerations (Fig. 2 d & e). Here we have not seen any browning of explants due to phenolic substances. This is probably because young seedlings do not synthesize phenolic compounds [26]. Earlier plant regeneration from seedling derived explants for of medicinal trees was reported in *Hildegardia populifolia* [27]. We have found that juvenile explants derived from two weeks old seedlings are ideal for *in vitro* cultures.

Embryo Axis Cultures

The efficient of embryo axis explants was studied under *in vitro* conditions. The position of the embryos in the medium played crucial in shooting response and multiple shoots development. The vertically oriented embryo axis in medium showed better response then the horizontally placed explants (Fig. 2 f). 70% maximal shooting response was observed on a medium containing 2.0 mg/L^{-1} BAP (Table 1). An average mean number of shoots per explants was 3.0±0.50 and with average mean shoot height of 7.1±0.04. BAP at other higher and lower concentrations are poorly responded. A couple of mean shoots were obtained on medium with 0.5 mg/L^{-1} kinetin with lower (30%) shooting response. In tree species embryo axis are ideal explants are commonly used in shoot regeneration was reported in previous research work [28].

Cotyledonary Nodal Cultures

The nature of cotyledons was increased many-folds from its original size after nutrients imbibitions in the medium after 14 days incubation. Shoot initiation was observed directly from cotyledonary node without intervening callus within 28 days cultures (Fig. 2 g). A maximum number of shootlets (4.2±1.80) was obtained on medium augmented with 1.5 mg/L^{-1} BAP 59% shooting responses followed by 3.0±0.99 shoots number obtained at 2 mg/L^{-1} with 48% response. The regenerated elongated higher shoot length (4.0±0.98) was also recorded. Kinetin at 1.0 mg/L^{-1} showed mean shoots of 2.8±1.23 was obtained (Table 1). For further shoot proliferations and elongation, the shoots were re-cultured at every four weeks intervals were achieved with BAP optimal concentrations. In our study concur with earlier studies conducted in several other woody tree species [29, 30]. Frequently, cotyledonary node are routinely used tissue explants in tree species *Syzygium densiflorum* [31] was reported.

Plant rooting, transplanting and acclimatization

For *in vitro* rhizogenesis, healthy shoots (5-7 cm length) from various explants were separated from shoot clusters were inoculated to the full strength woody plant (WP) medium for root induction. Three auxins (IBA, IAA & NAA) were tested individually along the medium for root development. No rooting response was seen in hormone free control medium. More than one root was produced when the shoots were cultured with various concentrations of auxins. In all the treatments, the root induction was observed in two weeks but complete root development took 6 weeks. The maximum of 90% rooting response noticed on medium augmented at 0.5 mg/L^{-1} IBA. A maximal roots number (4.9±0.98) and length (5.0±0.10) followed by 3.2±0.55 root number on medium at 0.1 mg/L^{-1} IBA were recorded (Table 2). Roots are very long, thick and healthy in nature showed better survival rate. The medium with NAA produces roots in 2 weeks culture. The rooting response was obtained at 1.0 mg/L^{-1} mean root numbers (4.3±1.17) with length of 4.9±0.37. The roots produce by NAA was very thin, fragile and weak in nature. Among the tested auxins, the IBA showed superior to IAA & NAA. Optimum root formation using IBA has also been employed effective rooting and higher survival rate was recorded for other woody plant species viz., *Santalum album* [15], *Punica granatum* [32]. The rooted *in vitro* plantlets were transferred to polythene bags containing autoclaved humus, red soil, and farmyard manure (1:1:1 ratio) for acclimation (Fig. 2 h) at 25 ± 2°C. Later, these plantlets were finally transferred to natural habitat after four weeks. About 70% survival rate was recorded. The field grown acclimatized plants showed normal in growth characteristics as like mother plants.

Table 1: *In vitro* direct shoot regeneration of seedling derived explants of *B. lanzan* cultured on WP medium.

Name of the Explants	Cytokinins (mg/L^{-1})		Shooting Response (%)	Mean No. of shootlets	Mean height of shootlets (cm)
	BAP	KIN			
Immature embryos	0.0		00	0.0	0.0
	0.1		39	2.0±0.15^{b}	2.6±0.03^{b}
	0.5		54	3.9±1.05^{cd}	4.2±0.06^{d}
	1.5		76	5.8±1.58^{e}	5.2±1.25^{de}
	2.0		68	4.3±0.08^{d}	4.8±0.08^{cd}
		0.5	60	1.5±0.60^{a}	2.9±1.40^{bc}
		1.5	48	1.0±0.79^{a}	1.6±1.40^{a}
Embryo axis	0.5		36	1.0±0.17^{a}	0.75±0.07^{a}
	1.5		59	2.0±1.25^{b}	1.20±0.12^{a}
	2.0		70	3.0±0.50^{cd}	7.1±0.04^{e}
	3.0		30	1.0±0.09^{a}	2.9±0.12^{bc}
		0.5	30	2.0±0.02^{a}	1.7±0.12^{a}
		1.0	28	1.8±0.12^{a}	1.0±0.09^{a}
Cotyledonary node	0.5		28	1.0±0.90^{a}	1.2±0.50^{a}
	1.0		40	2.3±0.70^{b}	2.4±0.03^{b}
	1.5		59	4.2±1.80^{d}	4.0±0.98^{d}
	2.0		48	3.0±0.99^{c}	3.5±0.06^{bc}
	2.5		22	2.1±0.12^{b}	2.7±0.07^{bc}
		0.5	18	1.0±1.04^{a}	1.0±0.66^{a}
		1.0	58	2.8±1.23^{a}	2.0±0.68^{a}

*Mean ± SD; mean for each experiment marked with the same letter does not differ significantly ($P < 0.05$)

Table 2 Effect of various auxins on root formation of *B. lanzan* in WP medium

Auxins (mg/L^{-1})			Rooting response (%)	Mean number of roots per explant	Mean root height (cm)
IBA	NAA	IAA			
0.0	0.0	0.0	00	0.0	0.0
0.1			65	3.2±0.55^{c}	3.8±0.09^{c}
0.5			90	4.9±0.98^{d}	5.0±0.10^{de}
1.0			79	2.8±1.4^{b}	3.0±0.13^{c}
1.5			61	1.6±1.20^{a}	2.1±0.33^{b}
2.0			43	1.0±0.05^{a}	1.0±0.45^{a}
	0.1		38	1.9±1.24^{a}	2.0±0.92^{b}
	0.5		62	2.5±0.61^{b}	2.7±0.87^{bc}
	1.0		80	4.3±1.17^{d}	4.9±0.37^{d}
	1.5		70	3.8±0.02^{c}	4.0±0.05^{cd}
	2.0		44	2.5±1.16^{b}	2.9±1.78^{bc}
		0.05	40	1.5±0.11^{a}	1.5±0.08^{a}
		0.5	51	2.7±0.31^{bc}	2.8±0.97^{bc}
		1.0	48	2.1±0.71^{b}	2.3±0.47^{b}

*Mean ± SD; mean for each experiment marked with the same letter does not differ significantly ($P < 0.05$)

CONCLUSION

In conclusion, we have developed an improved and reliable protocol for the regeneration of *B. lanzan* through embryo rescue techniques. The present study streamlined the optimized procedure, hormonal regimes and culture conditions were identified for maximal embryo germination and *in vitro* propagation from poorly developed seedling-derived embryo explants. Further, suitable strategies of plant acclimatization were also optimized for higher survival rate in the field. This protocol can be useful for the *ex-situ* conservation and rehabilitation of *B. lanzan* in the natural habitat. Further, the streamlined method can be useful for the mass propagation of plant material needed for the pharmaceutical industries.

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COMPETING INTERESTS

Authors do not have any conflict of interest.

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