EVALUATION OF LEAFY PART OF *Borreria verticillata* (L) (RUBIACEAE) CRUDE EXTRACTS FOR ANTI-DERMATOPHYTIC PROPERTIES AND ANTI DRUG RESISTANT PATHOGENS

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Abstract: The goal of this study is the search for more effective antimicrobial agents among materials of plant origin and also to discover potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs. Extracts from the leaves of *Borreria verticillata* were screened for their antimicrobial activities. Solvents used included hexane, chloroform, ethyl acetate, methanol and aqueous solvents. The *Borreria verticillata plant* leaves were air dried and powdered before being soaked in solvents for 3 days. The extracts were tested for the presence of different phytochemicals qualitatively, and were also tested against some Gram-positive organisms (*Staphylococcus aureus, Bacillus subtilis),* Gram-negative organisms (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi)* and some fungi implicated in dermatophytic infections (*Trichophyton mentagrophytes, Trichophyton rubrum, Microsporum canis, Epidermophyton flocossum*). Agar well diffusion and broth dilution methods were used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) at concentrations of 512 mg/mL to 4 mg/mL. The results showed that the yields of the extracts (g) ranged from 2.82 to 5.10 with the highest yield in the hexane extracts and the lowest yields in the aqueous extracts which was due to the decrease level in the order of polarity in the solvents. It was also noted that there was presence of some active ingredients in all of the crude extracts of the *Borreria verticillata* leaves and a considerable level of antimicrobial activities was observed in the results. The antimicrobial zone of inhibition ranged from 4.00 mm to 19.33 mm while the minimum inhibitory concentration (MIC) ranged from 32 mg/mL to 512 mg/mL and minimum bactericidal/fungicidal concentration (MBC/MFC) ranged from 128 mg/mL to 512 mg/mL. Randomised complete block design was used to determine whether there exist any significant differences among the treatment means of the antimicrobial activity of the leaves of *Borreria verticillata.*

Keywords: *Borreria verticillata*, crude extracts, anti-drug resistant pathogens, anti-dermatophytic properties

Introduction

The use and misuse of antimicrobials in human medicine and animal husbandry over the past 70 years has led to a relentless rise in the number and types of microorganisms resistant to these medicines - leading to death, increased suffering and disability, and higher healthcare costs (WHO, 2010). Drug resistance is the reduction in effectiveness of a drug such as an antimicrobial, anthelmintic or an antineoplastic in curing a disease or condition. More commonly, the term is used in the context of resistance that pathogens have "acquired", that is, resistance has evolved. When an organism is resistant to more than one drug, it is said to be multidrug-resistant (Gillespie and McHugh, 1997). It is for this reason of drug resistance in most microbes to common antibiotics that much emphasis is placed on the use of medicinal plant therapy; A traditional medicine plant is defined as any plant, which in one or more of its organs, contains substances that can be used for therapeutic purposes or which are precursors for synthesis of useful drugs (Sofowora, 1993).

Medicinal plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids, phenols and quinones (Cowan, 1999; Leon *et al.,* 2001; Al-Zubaydi *et al.,* 2009) which have been used worldwide in traditional medicine to treat several diseases and infection (Jain *et al.*, 1996; Kalemba and Kunicka, 2003; Saad *et al.,* 2005). Many studies all over the world have showed that these medicinal plants and their extract have multi-antimicrobial properties (Bocanegra-Garcia, 2009; Boklari, 2009; Al-Juraifani, 2011; Bakht, 2011). While 25 to 50% of current pharmaceuticals are derived from plants, none is used as antimicrobials (Cowan, 1999).

Historically, plants have provided a source of inspiration for novel drug compounds as plants derived medicines have made large contributions to human health and well-being (Achan *et al.*, 1980). The primary benefits of using plant-derived medicines in healing are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatments. Today, phytochemists and pharmaceutical companies depend on these medicinal plants (Kudi and Myint, 1999). Most people in the rural areas of the world depend largely on herbs for treatment of several ailments because medicinal herbs constitute indispensable components of traditional medicine practice due to low cost, easy access and ancestral experience (WHO, 2010). This has also been observed among the indigenes of Benue State, Nigeria. In some cases, it's been observed that herbal therapy is used alongside basic medical practices.

This study was validated by its attempt to confirming the veracity of the herbal practitioner's claim of this plant *(Borreria verticilata)* on pathogens most especially those implicated in dermatophytic infections and drug resistant microbes of clinical importance. *B. verticillata* (L.) (Syn.: *Spermacoce verticillata* L.), known in Brazil as "Poaia", in Nigeria, as Karya garma (Hausa), Wantiyo kporou (TIV), Irawo-ile (Yoruba), Abia-ikana (Ibibio) is a small perene and erect herb, originating from South and Central Americas and distributed by the Old World, Southern United States to South America (Vieira *et al.*, 1999; Chiquieri *et al.,* 2004; Ushie *et al.*, 2013).

In Brazil, the infusion of the flowers is used as antipyretic and analgesic (Vieira *et al.,* 1999; Moreira *et al.,* 2010) the roots as emetic and leaves as antidiarrheal, and for treat erysipelas and hemorrhoids (Lorenzi and Matos, 2002). In West India, the decoction of this plant is used for diabetes and dysmenorrhea, and when prepared with Cuscuta and Zebrina Schnizlein is used for amenorrhea (Ayensu, 1978); while in Senegal it is used to treat bacterial skin infections and leprosy (Maynart *et al.,* 1981). In Nigeria, fresh aerial part juice is applied for eczema (Benjamin, 1975) and in Jamaica the decoction of the endocarp, p repared jointly with Iresine P. Browne. and Desmodium, is used as a diuretic and as a remedy for amenorrhea mixed with Cuscuta and Zebrina (Asprey, 1955). The juice obtained from aerial part of the plant is applied topically for the treatment of skin diseases. A lotion is prepared to relieve skin itches (Liogier, 1990). In Gambia a lotion of the plant is used for febrile children. An essential oil extracted from leaves has been shown to inhibit *Escherícha coli* and *Staphylococcus aureus* (Burkill, 2000). It is employed in the form of enema for infantile hyperpnexia and treatment of leprosy, furuncles, ulcers, gonorrheal sores, biharzia and paralysis (Sofowora, 1982; Ushie *et al.,* 2013).

The study aimed at screening the antimicrobial activity of the leaves of *Borreria verticillata* against some pathogenic bacteria that are multi drug resistant and some dermatophytes while the specific objectives include the identification of the phytochemical constituents of the various parts of *Borreria verticillata* plant in various solvents, To confirm or disprove the efficacy of the various plant part extracts by evaluating their anti-dermatophytic and anti-drug resistant microbial activities.

Materials and Methods

Sample collection and preparation

Plant material

Borreria verticillata leaves were collected from Ucha village, a village adjacent to the University of Agriculture, Makurdi Local Government of Benue State, Nigeria. A quality evaluation of the plant material was carried out in the Department of Biological Sciences, University of Agriculture, Makurdi.

Sample preparation

The *Borreria verticillata* plant which was readily available in the rainy season was uprooted from the soil. The *B. verticillata* leaves were washed with running tap water to remove dirty prior to drying process. The sample was cut into small pieces and air died for 21 days to reduce moisture content and grinded into power with the aid of a pestol and mortar.

Extraction of plants material

Maceration method was employed for the extraction of plant active constituents. Marceration of the *Borreria verticillata* leaves were done by air-drying for two weeks and milled into fine powder using a Thomas-Willey milling machine. Aqueous solution of the milled plant parts was prepared by soaking 100 g of each in 250 ml hexane for four days. The resulting mixture was subjected to gravity filtration and the filtrates were concentrated by evaporation in a water bath, dried and weighed. The procedure was repeated on the residue using the following solvents: Hexane, ethyl acetate, chloroform and methanol sequentially in order of polarity. The extracts were stored in desiccators (Ushie *et al.,* 2010).

Phytochemical assay

Preliminary phytochemical screenings were carried out on the crude extracts as described by Brain and Turner (1975), Sofowora (1993), Edeoga *et al.* (2005), Harborne (1973), Okoli *et al.* (2010) and Ushie *et al*. (2010) to identify the presence of the classes of secondary metabolites (Alkaloids, flavonoids, tannins, saponins, glycosides, cardiac glycosides, terpenes, steroids, phenol).

Test for alkaloids

The extract (0.5 g) was stirred with 2 M aqueous hydrochloric acid (5.0 mL) on a steam bath. 1.0 mL of the filtrate were separately treated with a few drops of Mayer's reagent, Drangendoffs' reagent, Wagner's reagent. The resulting solution was observed for colour changes.

Test for tannins

0.5 g of each of the plant extracts was boiled with distilled water (100 mL) for 5 min. To 2.0 mL of the cooled solution (filtrate) a few drops of ferric chloride was added. The colour change was recorded.

Test for glycosides

A small portion of each of the plant extracts was placed in two separate test tubes of 0.1 M H_2SO_4 was added to one and distilled water (5.0 mL) added to the other. The test tubes were heated for 45 min in a water bath. The cooled solutions were made alkaline with a solution of 2M NaOH. Fehling solutions (5.0 mL) A and B (ratio1:1) was added to the two test tubes and were allowed to stand for 3 min. The solution of the extracts in distilled water serves as control. The changes in reaction were observed and recorded.

Test for saponins

The froth test and emulsion test as described by Harborne (1975) were used to determine the presence of saponins. A small portion of each of the plant extracts was added to distilled water (20 mL) in a 100 mL beaker, boiled and filtered and the filtrate used for the test; (a) Froth test: 5 ml of the filtrate was diluted with water (20 mL) and shaken vigorously and allowed to stand for 30 min. The result was recorded. (b) Emulsion test: 2 drops of olive was added to the frothing solution and shaken vigorously. The result was recorded. In order to remove 'false-positive", the blood haemolysis test was performed on the extract that frothed water.

Test for anthraquinones

0.5 g of each of the plant extracts was shaken with benzene (2.0 mL) and filter where necessary. 10 % ammonia solution (4.0 mL) was added to the filtrate. The resultant mixture was shaken and the reaction observed and recorded.

Test for flavonoids

(a) Lead acetate test: 0.5 g of the extract dissolved in 5 mL of distilled water. 10 % of lead acetate solution (1.0 mL) was added. The colour formation was recorded. (b) Iron (III) chloride. To a solution of 0.5 g of the extract in water, two drops of iron (III) chloride was added. A colour change noted and recorded.

Test for terpenoids (Salkowski test)

A solution of each of the extract was made by dissolving 0.5 g of the extract in 2.0 mL of chloroform and concentrated H_2SO_4. The presence of terpenes in the sample was detected as the colour changes.

Source and maintenance of organisms

Gram-positive organisms (*Staphylococcus aureus, Bacillus subtilis)* and Gram-Negative organisms (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Escherichia coli*, *Salmonella typhi)* were obtained and confirmed resistant to at least two of the convectional antibiotics such as chloramphenicol, ampicillin and cotromoxazole at the Medical Microbiology and Parasitology unit of the Clinical Laboratory Department of the Federal Medical Centre, Keffi using the antibiogram susceptibility tests. The fungi implicated in dermatophytic infections (*T. mentagrophytes, T. rubrum, Microsporum canis, E. flocossum*) were obtained at the Medical Microbiology and Parasitology department of the Ahmadu Bello University Teaching Hospital. They were maintained on nutrient agar and Sarbaround's dextrose agar respectively (Oxoid, UK). Twenty-four hours old pure cultures were prepared for use each time.

Antimicrobial aioassay

The bacterial assay procedures of Water Worth (1978) and Perez *et al*. (1990) was employed with small modification (Ushie *et al*., 2012). The methods involved the preparation of the culture medium and inoculation. Aseptic technique was used to avoid contamination (Ushie *et al*., 2012). The agar plates were inoculated by spreading a small volume (0.05 mL to 0.10 mL) of the liquid inoculums (sub-cultured nutrient broth) by means of an L-shaped glass rod in such a way that the surface of the agar in the plates was covered with microbes. One microbe was inoculated to one plate making a total of ten plates for ten microbes.

Five wells for hexane, chloroform, ethyl acetate, acetone, and methanol extracts and two for the control were made through the aid of a sterile cork borer. The plant extracts were diluted using dilution method and in each of the appropriately labelled well (hole) diluted plant extract was introduced. Ciprofloxacin and fulcin were introduced in the other two wells (holes) as control. The inoculated plate was left on the bench for about an hour to allow the extracts diffuse into the agar. The plates were aerobically incubated at 37^{o}C for 23 h for the bacteria and 72 h for the fungi. The diameter of zones of inhibition was measured by means of linear instrument in millimeter (venier calliper) and recorded (Akinyemi *et al.*, 2005).

Determination of the minimum inhibitory concentration (MIC)

To measure the MIC values, suspension of micro-organisms were made in sterile normal saline and adjusted to 0.5 Macfarland standard (10^{8} Cfu/mL) (NCCLS, 2000). From the stock solution, serial dilutions were made to 512, 256, 128, 64, 32, 16, 8, 4 mg/mL (NCCLS, 2000). The various concentrations of the stock were prepared in about different test tubes labelled 1-8, respectively. These were assayed against the test bacteria. The minimum inhibitory concentration was defined as the lowest concentration able to inhibit any visible bacterial growth (Prescott *et al*., 1999; Shahidi Bonjar, 2004).

Determination of minimum bactericidal/fungicidal concentration (MBC/MFC)

This was an offshoot of the previously determined MIC. Equal volume of the various concentrations of each extract and Sarbaround's dextrose agar (Oxoid, UK) were mixed in micro-tubes to make up 0.5 mL of solution. 0.5 mL of McFarland standard of the organism suspension was added to each tube (Shahidi Bonjar, 2004). The tubes were incubated aerobically at 37^{o}C for 24 h for MDR-bacteria, and 72 h for dermatophytes. These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller Hinton Agar and further incubated for 24 h. The highest dilution that yielded no single bacterial/fungal colony was taken as the Minimum bactericidal/fungicidal Concentration (Akinyemi *et al.*, 2005). This was carried out on some of the extracts with high antimicrobial activity and some of the highly sensitive organisms.

Statistical analysis

Data obtained were subjected to analysis of variance and means separated according to Duncan's Multiple Range Test at $P = 0.05$. Randomised complete block design was used to determine whether there existed any significant differences among the treatment means of the antimicrobial activity of *Borreria verticillata* plant leaves.

Results and Discussion

Table 1 shows the nature and yield of different solvents extract of the *Borreria verticillata* leaves. The yield of the extracts is higher in hexane and lowest in the aqueous medium according to the polarity of the solvents used. It was observed that of the five solvents extracts used, the hexane extract gave a higher yield while the lowest yield was recorded in the aqueous extract.

Table 1: Nature and yield of different solvents extract of the leaves of *Borreria verticillata*

Solvents	Colour of Extract	Texture of Extract	Yield of Extract (g)
hexane	Brownish	Hard Solid	5.10
Ethyl acetate	Dark brown	Sticky solid	3.90
Chloroform	Light Brown	Powder	3.85
Methanol	Light Brown	Powder	3.45
Aqueous	Light Brown	Powder	2.82

Results of the qualitative phytochemical screening of the crude extract of *Borreria verticillata* plant leaves were presented in Table 2. The antimicrobial activities of the extracts obtained from *Borreria verticillata* leaves, using different solvents and extracts against the tested organisms were shown in Table 3. The zones of inhibition of each organism are presented in the aforementioned Table. The minimum inhibitory concentrations of the extracts of the leaves of the *BVR* plant leaves which ranged between 32 mg/mL to 512 mg/mL are shown in Table 4. Table 5 shows the minimum bactericidal/fungicidal concentrations of some of the most active extracts, which rangeg between 128 mg/mL to 512 mg/mL.

Table 2: Result of the qualitative phytochemical screening of the crude extract of *Borreria verticillata* leaves

Phytochemicals	Reagents	Extracts				
		HE	EAE	CE	AE	ME
Alkaloids	a) Wagners	–	–	–	–	–
	b) Mayer	–	–	–	–	–
	c) Drangedroff	–	–	–	–	–
Tannins	Solutions of extracts plus ammonia solution	-	-	-	-	+
Flavonoids	a) Lead acetate	-	+	-	-	+
	b) Ferric chloride	+	+	–	–	+
Anthraquinone	Extract in benzene plus ammonia solution	-	-	-	-	-
Terpenes	Extracts plus chloroform plus H_2SO_4	+	-	-	+	+
Saponins	a) Frothy test	+	+	-	+	+
	b) Emulsion test	+	–	+	–	+
Glycosides	Extracts plus dilute H_2SO_4 plus NaOH plus Fehiling solution	+	+	+	+	+

– = Absent; + = Present; HE= Hexane extract; AE=Aqueous extract; CE=Chloroform extracts; EAE= Ethyl acetate extracts; ME= Methanol extracts.

Table 3: Diameter of zone of inhibition of the antimicrobial activity of crude extract in mm of *Borreria verticillata* leaves on selected MDR bacteria strains and some dermatophytes

Test organisms	HE	CE	EAE	AE	ME	CPN	FCN
S. aureus	11.00b,c	11.67b	8.00d	8.63c,d	12.67b	25.33a	NA
E. coli	9.00d	6.57e	7.97d,e	11.00c	15.00b	23.67a	NA
B. subtilis	4.00c	5.00c	8.00b,c	7.67b,c	19.33a	22.67a	NA
S. typhi	5.67c	8.33c	6.67c	6.67c	8.33c	23.33a	NA
P. aeroginosa	6.67d	4.67d	10.33c	10.33c	14.00b	22.67a	NA
K. pneumoniae	9.00b	8.00b	9.00b	10.67b	7.67b	23.00a	NA
M. canis	8.33b	5.67b	5.00b	6.00b	4.67b	NA	23.33a
T. rubrum	7.00c	2.67d	5.00c,d	4.67c,d	7.67c	NA	26.00a
E. flocosum	4.00b	6.33b	9.00b	8.67b	10.33b	NA	23.00a
T. mentagrophytes	8.67c	8.33c	7.67c	7.67c	9.67b,c	NA	22.67a

Data are means of three replicates. Means followed by the same letter in each vertical column are not significantly different while means followed by different letter in each vertical column are significantly different according to Duncan's multiple range test (*P* = 0.05). – Absent; + Present; HE= Hexane extract; CE=Chloroform extracts, EAE= Ethyl acetate extracts, AE=Aqueous extract, ME= Methanol extracts. NA= Not Applicable.

The preliminary phytochemical screening of the crude extracts of the *Borreria verticillata* leaves revealed the presence of tannins, flavonoids, saponnins, terpenes and glycosides in some of the extracts. The chemical test shows the absence of anthraquinones and alkaloids in all the extracts. This confirms the assertion that the *Borreria verticlata* plant leaves can be use used for medicinal purpose. Medicinal plants have always been known to contain active principles which are phytochemicals with biological activity, some of which are responsible for the characteristic odours, pungencies and colours of plants while others give a particular plant its culinary, medicinal or poisonous virtues (Evans, 2002; Sofowara, 1993; Ushie *et al.*, 2013).

The phytochemical screening of the crude yields of the chemical constituents of *Borreria verticillata* leaves showed the presence of some secondary metabolites that are known to show medicinal activity as well as exhibiting physiological activity (Sofowara, 1993; Ushie *et al.*, 2013). The presence of these secondary metabolite in any plant have been known to give the plant anti-allergic, anti-inflammatory and antimicrobial properties (Cushnie and Lamb, 2005). They are also antioxidants and free radical scavengers which prevent cell damage, and have strong anticancer activity and protect the cell against carcinogenesis (Saleh *et al.*, 1995; Okwu, 2004). Hence, the establishment of the fact that the plant can be of immense value to the medical, pharmaceutical and cosmetic industry (George, 1965). For instance, saponins as a secondary metabolite have been known to be present in traditional medicine preparations (Xu *et al.*, 1996).

The antimicrobial activities of the extracts were tested against some clinical isolates (MDR-bacteria strain) and dermatophytes (fungi implicated in skin surface related infections). The antimicrobial activity of the different extracts of the leaves of *Borreria verticillata* were tested against the growth of the selected isolates. The results showed that the plant leaves possess antimicrobial activity at the particular concentration used, this also agreed with the research findings of Sofowora (1982); Benjamin (1975) pointed out that *BVR possess* antimicrobial action at different concentration depending on the bacteria species.

It could be observed that the *Borreria verticlillata* plant leaves possess relatively good antimicrobial properties even in different solvents extracts. Cheesbrough (2000) pointed out that the active antimicrobial compound diffuses from the disc into the medium and the susceptible organisms are inhibited at a distance from the disc. This was clearly shown in the comparism of the results with well known antibiotics that shows broad spectrum against both gram positive and gram negative bacteria and anti fungal drug; that is ciprofloxacin and fulcin, respectively.

Table 4: Minimum inhibitory concentration (MIC) in mg/mL of the crude extract of the *Borreria verticillata* leaves

Extracts	*S. aureus*	*E. coli*	*B. subtilis*	*S. typhi*	*K. pneumoniae*	*P. aeruginosa*	*T. mentagrophytes*	*T. rubrum*	*E. floccosu*	*M. canis*
HE	128b	128b	256b	512a	128b	32b	128a		-	-
CE	256b	128b	256b	-	128b	32b	-	512a	-	-
EAE	512a	512a	256b	-	128b	32b	-	512a	-	-
AE	128b	128b	256b	512a	128b	32b	8b		-	-
ME	256b	-	512a	-	512a	256a	-		256a	-

Data are means of three replicates. Means followed by the same letter in each vertical column are not significantly different while means followed by different letter in each vertical column are significantly different according to Duncan's multiple range test (*P* = 0.05); HE = Hexane extracts; AE = Aqueous extracts, CE = Chloroform extracts, EAE = Ethyl acetate extracts, ME = Methanol extracts.

Table 5: Minimum bactericidal/fungicidal concentration (MBC/MFC) in mg/mL of the crude extract of the *Borreria verticillata* leaves

Extracts	*S. aureus*	*E. coli*	*B. subtilis*	*S. typhi*	*K. Pneumoniae*	*P. Aeruginosa*	*T. Mentagrophytes*	*T. rubrum*	*E. floccosum*	*M. canis*
HE	-	-	-	256a	-	-	-	-		-
CE	128b	-	-	-	256a	-	128a	-	-	-
EAE		-	256	-	512a	-		-		-
AE	512a	-	-	-	-	256a	-	-	-	-
ME	-	256a	-	-	512a	-	-	512a		-

Data are means of three replicates. Means followed by the same letter in each vertical column are not significantly different while means followed by different letter in each vertical column are significantly different according to Duncan's multiple range test (*P* = 0.05). HE = Hexane extract; AE = Aqueous extract; CE = Chloroform extracts; EAE = Ethyl acetate extracts; ME = Methanol extracts.

The MIC (minimum inhibitory concentration), MBC/MFC (minimum bacteriacidal/fungicidal concentration) of the crude extracts from the leaves of the *Borreria verticillata* plant proved that the plant has a relatively significant anti drug-resistant bacterial and anti dermatophytes activity (Benjamin, 1975). Just like other species of the Spermacoce and Borreria genus have been found to demonstrate anti drug resistant bacterial and even antifungal (Taylor, 2004). However, It is worthy of note that MBC values obtained for the extracts against the pathogens are higher than MIC, indicating that the extracts are bacteriostatics at lower concentrations and bactericidal at higher concentrations. This suggests that these plant extracts, when used traditionally as antimicrobials inhibit bacteria growth without necessarily killing the bacteria and since most of the traditional preparations lack specific concentrations, this may thus account for the use of large quantity of the extracts by traditional medical practitioners for the treatment of their patients (Akinyemi *et al.,* 2006).

Conclusion

The present investigation confirms the folkloric use of the *Borreria verticillata* plant leaves, as indigenous medicine for the treatment of some bacteria and fungi associated diseases in different parts of the world and that the different parts of the plant can indeed be used to combat multi drug resistant bacteria pathogens and dermatophytic related infections. The plant also contains important bioactive substances (phytochemicals) which can be produced in large quantity for commercial purposes. Some of the importance of these bioactive substances have been highlighted and outlined earlier in the present study. These could really be exploited by basically the cosmetics and the pharmaceutical industries.

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Conflict of Interest

The authors declare that there are no conflict of interest.

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