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Antifungal Activity of Nine Medicinal Plants against *Aspergillus* species from Cocoa Beans (*Theobroma cacao*)

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Authors' contributions

This work was carried out in collaboration between all authors. Author OOO designed the study, wrote the protocol, compiled the literature review and wrote the first draft of the manuscript. Author RTO participated in the experimentation section while author TOA proof read the manuscript. All the authors read the final manuscript.

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ABSTRACT

Aims: The study was to screen the activities of nine medicinal plants against *Aspergillus* species isolated from stored cocoa bean seeds collected in Akure, Owo, Ondo, Ile-Oluji, Ikpenmen, and Oba-Akoko, all in Ondo State, Nigeria with a view to getting a potent, cheap antifungal plant that is easily available and capable of halting fungal infestation and subsequent mycotoxin production.
Place and Duration: The study was carried out in Adekunle Ajasin University, Akungba-Akoko between July, 2014 to November, 2014.
Methodology: Inhibitory activities of locally sourced plant parts were tested against nine *Aspergillus* species isolated from cocoa bean seeds from Ondo State, Nigeria. Methanolic extraction of the plant parts yielded crude extracts which were tested against the fungal species using the food poison method and filter disc methods.

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Results: Extraction of phytochemicals showed that sweet potato had the highest yield of 13% with a creamy coloration. Radial growth of *Aspergillus foetidus* was 12 mm at 24 h incubation while *A. niger* aggregate and *A. aculeatus* almost overgrew their plates at 72 h with 42 mm radii. The extracts of bitter leaf did not appreciably stop growth of the fungi as it did not inhibit *A. niger* aggregate, *A. aculeatus, A. nidulans, A. carbonarius* and *A. glaucus. Lippia alba* resisted the growth of all the fungi at very high percentages except *A. fumigatus* at 10±0.9% inhibition.
Conclusion: The study showed that plant extracts possesses antifungal potentials and so their continued use in traditional everyday medicine justified.

Keywords: Aspergillus; mycotoxin; extraction; phytochemicals; antifungal.

1. INTRODUCTION

The use of plant parts for various purposes dated to the early ages [1]. Till today, plant parts are used for curative medicine and preventive purposes [2]. Phytochemicals have an array of important active components which can be derived from any part of plant such as seeds, leaves, bark, flower, roots [1,3]. The aforementioned active compounds differ in plants depending on their growing conditions, variety, and age at harvest, extraction methods and storage conditions [4].

Plant parts contain secondary metabolites including organic compounds that are involved in growth and development processes of plants. Lately, efforts are being made to develop potent drugs of plant origin that can prevent and cure diseases, as well as tackling pathogen resistance. To these end, resulting drugs should be generally accepted, cost effective and not produce any residual toxic components. Plants possess phytochemicals that are abundant, renewable, and environmentally safe, with antimicrobial properties that endear people to their uses [5].

Contamination and degradation of agricultural crops and products by fungi, especially *Aspergillus* species have posed great challenges to food safety in Nigeria and Africa [6]. *Aspergillus* and *Penicillium* species are generally classified as storage fungi [7]. These fungi are capable of growing at low water contents than the field fungi, and they tend to contaminate grains in silos and in other storage containers [8]. *Aspergillus niger* and *A. ochraceus* have been implicated in the production of ochratoxin A and B (OTA and OTB). OTA is a fungal metabolite produced during the fungal growth and development on agricultural crops and products, and it has been classified as a carcinogen [9]. The research aims to screen activities of nine medicinal plants against *Aspergillus* species isolated from cocoa bean seeds in Akure, Owo, Ondo, Ile-Oluji, Ikpenmen, and Oba-Akoko, all in Ondo State, Nigeria with a view to getting a potent, cheap antifungal that is easily available and capable of halting fungal infestation and subsequent mycotoxin production.

2. EXPERIMENTAL DETAILS

2.1 Collection and Identification of Plant Materials

Nine (9) plant materials of traditional medicinal importance were sourced and tested for ability to inhibit fungal growth. The plant materials which include *Lippia alba*, *Cymbopogon citratus, Ganoderma lucidum, Ipomoea batatas, Vernonia amygdalina, Zingiber officinale, Thymus vulgaris, Moringa oleifera*, and *Garcinia kola* (Table 1) were collected from Akungba-Akoko and Iwaro-Oka in Akoko South-West and Arimogija Forestry Reserve, Ifon, both in Ondo State, Nigeria. They were identified and authenticated by the Plant Science and Biotechnology Department, Adekunle Ajasin University, Akungba-Akoko. After the identification, the plants were washed and dried under shade for twenty one (21) days, after which they were ground to powder form. The dried samples were separately blended into fine powder using a Marlex Electroline electric blender Model IS: 4250, CM/L 7371373, and were kept in polyethylene bags, labeled and stored in the refrigerator at 20℃.

Fresh fruiting bodies of *Ganoderma lucidum* were harvested, cleaned, shade dried, and ground into cottony form, and then kept in the refrigerator for further testing. Healthy leaves of *Lippia alba*, *Cymbopogon citratus, Vernonia amygdalina, Thymus vulgaris,* and *Moringa oleifera* were collected between August and September, 2014. Other plant materials were collected in October, 2014.

Table 1. List of plants and parts used

S/N	Botanical name	Local/common name	Medicinal importance	Part harvested	References
1	*Moringa oleifera*	Moringa (leave)	Anticancer agent, ulcer treatment	Leaves	[10]
2	*Cymbopogon citrates*	Lemon grass, ewe tea	Relieves nasal congetion	Leaves	[11]
3	*Ipomoea batatas*	Sweet potato, kukunduku	Anti-inflammatory agent,	Tuber	[12]
4	*Lippia* sp	Oganju		Leaves	
5	*Zingiber officinale*	Ginger	Relieves arthritis, stomach problems	Rhizome	[13]
6	*Vernonia amygdalina*	Bitter leaf, ewuro	Anticancer, antiviral, antioxidant	Leaves	[14]
7	*Ganoderma lucidum*	Reishi mushroom	Cancer and diabetes treatment	Fruiting bodies and gills	[15]
8	*Lippia alba*	Scent leaf	Relieves stomach pain	Leaves	[16]
9	*Garcinia kola*	Bitter kola, orogbo	Detoxify body, antihepatoxic	Seed	[17]

2.2 Extraction of Samples

The modified methods of Odey et al. [18] were employed in the extraction process as follows: Four hundred grams (400 g) each of the plant part was separately weighed and soaked in 2000 mL of methanol (98% BDH) at a ratio of 1:5 (powder: solvent). The mixtures were kept in corked or air-tight containers and left for 48 hr at room temperature.

Filtrations to remove residue were done using double layer muslin cloth followed by another stage of filtration using WhattMan No 1 filter paper (24 cm). The filtrate was then separately concentrated *in vacuo* using Rotary Evaporator (Model RE52A, China) to 10% of the original volume at 37℃ - 40℃. The final concentrations to dryness were done by evaporating to dryness in water bath at 60℃.

2.3 Determination of Extraction Yield (% yield)

The percentage yield (w/w) from the extraction process was calculated thus:

$$\text{Yield (\%)} = (W1 \times 100)/W2$$

Where

W1: weight of extract after dissolving with solvent,
W2: weight of dried plant powder.

2.4 Fungal Isolates

Twelve *Aspergillus* species isolated from stored cocoa bean seeds were used for the study. The isolates include *Aspergillus flavus* (LBFC299), *A. niger* (LBFC394), *A. niger* aggregate (LBFC396), *A. ochraceus* (LBFC271), *A. versicolor* (LBFC283), *A. ustus* (LBFC402), *A. aculeatus* (LBFC371), *A. fumigatus* (LBFC377), *A.foetidus* (LFC379), *A. nidulans* (LBFC393), *A. terreus* (LBFC402), and *A. carbonarius* (LBFC373). They were obtained from The Library of Bacteria and Fungi Collection (LBFC), Department of Microbiology, Federal University Lafia, Nasarawa State, Nigeria.

2.5 Fungal Inoculum Preparation

The fungal inocula were prepared using the modified methods of Geetha et al. [19] and Lachumy et al. [20]. Ten millilitre of sterile distilled water was added to 6 days old fungal colonies in Petri dishes containing fungal mycelia and shaken gently. The fluid containing fungal spores were collected with sterile pipette. The cell density was adjusted to 0.5 x McFarland standards by measuring the absorbance in a spectrophotometer at a wavelength of 530 nm, and adding sterile distilled water as required. The fungal suspensions corresponded to optical density (OD) of 0.08 and 0.13 on the spectrophotometer at 625 nm and 108 cfu/mL. The turbidity of overnight broth culture of fungi were compared with that of McFarland scale tubes against white background and concentration was approximated to the table.

2.6 Assay Test of Plant Extracts

2.6.1 Concentration of plant extracts for antifungal activities

Plant extracts were re-dissolved in methanol, which was the extracting solvent. The prepared extract solutions were sterilized using Millipore (0.22 µm pores) before the tests were carried out. One (1) gram of each extract was dissolved in 10 mL methanol to yield a concentration of 100 mg/mL.

2.6.2 Agar dilution technique

The modified methods of Kumar et al. [21] were used. The re-dissolved, sterilized, plant extracts were mixed with sterile Potato Dextrose Agar medium (PDA) to obtain the final concentration of 100 mg/mL for each plant extract, and then poured into sterile Petri dishes (90 mm diameter). To avoid bacterial contamination, 0.5 g streptomycin was added to 1L of PDA.

For the control, 5 mL of Millipore-sterilized methanol was added to PDA, and 7 mm disc of fungi from the periphery of 6 day-old cultures were inoculated onto the center of the Petri dishes and incubated at 28°C for 7 days. Radial growths of each test organism in triplicates were recorded on a daily basis for 3 days. Plates containing methanol and fluconazole (10 µg/ml) served as negative and positive controls respectively. The percentage of mycelial inhibition was calculated thus:

$$\text{Percentage of mycelial inhibition} = [C - T / C] \times 100$$

Where, C and T are the growth diameter (mm) in control and treatment respectively.

2.6.3 Minimum inhibitory concentration

The minimum inhibitory concentrations of the antifungal plant extracts were obtained using the filter paper disc diffusion method. The methods of Lachumy et al. [20] were employed. Fifteen milliliter (15 mL) steriled Sabouraud Dextrose Agar (SDA) 45℃ was poured in 9 cm sterilized Petri dishes. Fungal inocula containing 108 cfu/mL spores were spread on the solid plates with inoculating loop. Sterile filter paper discs (6 mm diameter) individually impregnated with 40 mg/disc, 20 mg/disc and 10 mg/disc of each of the plant extracts were placed on the agar plates that had previously been inoculated with test fungi. Similarly, on each plate a blank disc impregnated with sterile methanol was used as negative control. All the plates were incubated at 28℃ for 48 h. The diameters of inhibition zones were measured in millimeters. All the tests were performed in triplicate.

2.7 Statistical Analysis

Statistical analyses of inhibition of radial growth were subjected to one way analysis of variance (ANOVA) using SPSS software. Tukey HSD All-Pairwise Comparisons Tests at 5% was used to compare the means.

3. RESULTS AND DISCUSSION

3.1 Yield of Phytochemicals

Sweet potato gave the highest yield of 13% after methanolic extraction (Table 2). The extract was creamy in color. Bitter kola and bitter leaf yielded 1% extracts each which were the lowest values with pink and greenish brown color.

3.2 Radial growth of *Aspergillus* species against Phytochemicals

Table 3 showed the result of 72 h antifungal inhibition of the *Aspergillus* species by plant extracts. The result showed that, *Lippia alba* resisted growth of *A. aculeatus, A. ochraceus, A. versicolor, A. carbonarius, A. ustus* and *A. glaucus.* Similar trend was observed in the extract of *Ganoderma lucidum*. Extracts of ginger, bitter leaf, *Lippia* sp, and *Moringa oleifera* all had little inhibitory activities against the fungi. *A fumigatu*s at 72 h had nearly overgrown plates incorporated with ginger and lemon grass (Table 3). *A. niger* aggregate and *A. aculeatus* resisted ginger, bitter leaf, sweet potato and the *Lippia* sp. to nearly overgrow their plates. *A. niger* aggregate also resisted *Moringa oleifera* leaf and *Ganoderma lucidum* extracts growing to 42 mm radii. *A. niger* aggregate radial growth was hindered by lemon grass and bitter kola with radii of 7 mm and 9 mm respectively while, *Lippia alba* and *Moringa oleifera* leaf extracts hindered the radial growths of *A. aculeatus* to 0 mm and 9 mm respectively.

3.3 Fungal Inhibition by Plant Extracts

As shown in Table 4, the minimum inhibitory concentrations of the extracts showed that at 40 mg/disc, ginger did not inhibit growth of *A. niger* and *A. ochraceus. A. aculeatus* were not inhibited at the highest concentration of 40 mg/disc tested against ginger, bitter leaf, potato tuber, bitter kola, and the *Lippia* sp.

Colonization of agricultural produce and by-products is a very serious problem in tropical warm regions of the world [22]. Storage fungi especially *Aspergillus* genera of the black moulds not only contaminate but produce mycotoxins which elicit disease conditions at different concentrations in man and animals [23].

Extract of ginger (*Zingiber officinale*) inhibited radial growth of *A. nidulans, A. versicolor*, and *A carbonarius* at over 59% inhibition (Table 5). The report is in agreement with earlier reports presented by Avasthi et al. [22] and El-Ahmedy et al. [24] in which ginger extract proved not potent to prevent growth of *A. niger*. The ineffectiveness of the extract was reported to be due to insolubility of active metabolites in the solvent [25]. Atai et al. [26] and Supreetha *et al*. [27] in separate reports opined that the presence of gingerol, an antioxidant in ginger confers antifungal properties against *Aspergillus* species [28]. Kubra et al. [29] added that the presence of dehydrozingerone in ginger with antifungal properties might be responsible for its activities against the *Aspergillus* species.

The activities of bitter kola (*Garcinia kola*) were not encouraging. Extract of the dried rhizome only showed promising activities against *A. niger*, and *A. fumigatus* at over 80% inhibition of fungal growth (Table 5). The extract inhibited *A. glaucus* and *A. carbonarius* at over 50% inhibition. The inhibitory activities of the extract could be due to the presence of benzophone and flavononesn present in the rhizome [30,31]. Farombi et al. [17] reported the presence of polyisoprenyl benzophenone (kalonone) in bitter kola which

Table 2. Percentage extract yield of medicinal plants

Plants	Percentage yield (%)	Color of extract
Moringa oleivera	3.0	Light green
Cymbopogon citrates	2.08	Brown
Ipomoea batatas	13	Cream
Lippia sp.	3.5	Yellowish green
Zingiber officinale	10	Dirty cream
Vernonia amygdalina	1.0	Greenish brown
Lippia alba	2.57	Green
Ganoderma lucidum	1.62	Brown
Garcinia kola	1.0	Pink

Table 3. Radial growth (mm) of *Aspergillus* species at 72 hours at concentration of 100 mg/mL

Extracts	A	B	C	D	E	F	G	H	I	J	K	L
Control	25^{cd}	36^{a}	42^{a}	33^{b}	27^{bc}	42^{a}	33^{b}	12^{d}	19^{d}	39^{a}	42^{a}	38^{a}
Ginger	42^{a}	20^{b}	42^{a}	42^{a}	21^{b}	9^{c}	11^{c}	2^{d}	8^{c}	8^{c}	24^{b}	37^{a}
Bitter leaf	42^{a}	21^{c}	42^{a}	19^{c}	12^{cd}	30^{a}	11^{cd}	6^{d}	8^{d}	36^{a}	6^{d}	10^{cd}
Sweet potato	42^{a}	12^{cd}	42^{a}	37^{a}	9^{d}	15^{c}	16^{c}	9^{d}	14^{c}	19^{c}	12^{cd}	29^{b}
Lippia sp.	42^{a}	20^{b}	42^{a}	6^{c}	8^{c}	42^{a}	13^{b}	6^{c}	15^{b}	10^{bc}	9^{c}	8^{c}
Bitter kola	9^{c}	9^{c}	29^{b}	4^{d}	23^{b}	42^{a}	9^{c}	7^{cd}	14^{c}	9^{c}	11^{c}	14^{c}
Lemon Grass	7^{d}	23^{c}	13^{c}	42^{a}	15^{c}	6d	33^{b}	11^{cd}	15^{c}	21^{c}	15^{c}	21^{c}
Moringa oleifera	42^{a}	17^{c}	9^{cd}	7^{d}	31^{b}	11^{c}	11^{c}	7^{d}	9^{cd}	1^{e}	7^{d}	0
Lippia alba	22^{b}	37^{a}	0	3^{d}	5.7^{d}	12^{c}	23^{b}	0	4^{d}	0	0	0
G. lucidum	42^{a}	16^{b}	8.7^{c}	8.1^{c}	7.7^{c}	5.3^{c}	10.7^{c}	0	0	6^{c}	0	8^{c}

Key: A: A. niger aggregate; B: A. niger; C: A. aculeatus; D: A. fumigatus; E: A. foetidus; F: A. nidulans; G: A. terreus; H: A. ochraceus; I: A. versicolor; J: A. carbonarius; K: A. ustus; L: A. glaucus.

a^{1}= Means with different letters are significantly different at P= 0.05 (Tukey HSD all-pairwise comparisons test)

confers the rhizome with its antimicrobial properties. Poor inhibitory potential of G. kola against yeast and bacteria had earlier been reported by Uzondu et al. [32]. The authors extracted active components of the plant with diethyl ether and tested it at different concentration against *Candida albican* and *Escherichia cola;* no inhibitions against the microorganisms were recorded.

Cymbopogon citratus (lemon grass) extract inhibited growth *A. niger* aggregate, *A. aculeatus*, mycelia and other *Aspergillus* species at over 40% (Table 5). The extract failed to inhibit growth of *A. fumigatus, A. ustus*, *A. terreus* and *A. glaucus*. Lemon grass contains alkaloids, tannins, cardiac glycosides, aliphatic alcohol, geranial, neral, aliphatic aldehydes [33-35]. Aliphatic alcohols and phenols in it were reported to possess antifungal activities [36], which might be responsible for its inhibitory activities. Our report agrees with the work of Kakarla and Ganjewala [37] who reported that the essential oils of lemon grass possess inhibitory activity on *A. flavus* and *A. fumigatus*. It could be inferred that the essential oils contain potent bioactive compounds which were not present in the methanolic extract. Another author further explained that antimicrobial properties and essential oil present in lemon grass is affected by such morphological feature of the microorganism such as the cell membrane [38]. Pawar and Thakar [39] and Yousef [40] in separate studies reported that lemon grass inhibit *A. niger* growth by disrupting cell membrane integrity and mitochondrial structure organization thus, restricting hyphal growth and spore formation.

Bitter leaf (*Vernonia amygdalina*) inhibited radial growth of *A. glaucus* but recorded no inhibition against *A. niger* aggregate, *A. aculeatus, A. nidulans, A. carbonarius* and *A. ustus* (Table 5). The inhibitions recorded against the fungi could be as a result of active components in the extract such as alkaloids, saponins, tannins, and glycosides. Suleiman et al. [41] and Tahany et al. [42] reported *V. amygdalina* as fungicidal to *Fusarium* species, while Agoramorthy et al. [43] explained that the fungicidal attribute of the leaf results from the presence of fatty acids and their methyl esters.

Table 4. Minimum inhibitory concentration (MIC) of plant extracts at different concentration per disc against *Aspergillus* species

Aspergillus species	Ginger	Bitter leaf	Potato tuber	Bitter kola	Lemon grass	*Lippia* sp	*M. olievera*	*L. alba*	*G. lucidum*
A. niger aggregate	NI	NI	NI	C	B	C	NI	A	A
A. aculeatus	NI	NI	NI	NI	B	NI	B	A	A
A. foetidus	C	B	A	C	C	NI	C	A	A
A. niger	NI	C	B	A	C	C	B	A	B
A. fumigatus	C	C	C	A	NI	A	NI	A	C
A. nidulan	C	NI	B	NI	A	NI	B	C	C
A. terreus	C	A	B	C	C	B	C	A	A
A. ochraceus	NI	A	B	NI	C	B	A	A	A
A. versicolor	C	A	B	C	C	C	A	A	A
A. carbonarius	C	NI	B	B	C	B	A	A	A
A. ustus	C	NI	A	C	NI	B	A	A	A
A. glaucus	NI	A	C	A	NI	B	A	A	A

KEY: A – 10 mg/disc extract; B – 20 mg/disc extract; C – 40 mg/disc extract; NI – No Inhibition

Table 5. Percentage inhibition of *Aspergillus* species by plant extracts

Aspergillus species	Ginger	Bitter leaf	Potato tuber	Bitter kola	Lemon grass	*Lippia* sp.	*G. lucidum*	*Moringa oleifera*	*Lippia alba*
A. niger aggregate	NI	NI	NI	3±1	55±21	39±2	87±14	NI	81±2
A. aculeatus	NI	NI	NI	NI	58±35	NI	62±19	67±26	100
A. foetidus	31±9	57±2	66±5	29±1	49±13	NI	76±12	3±1	80±2
A. niger	NI	44±31	51±28	80±4	42±10	42±10	54±5	56±7	62±9
A. fumigatus	4±2	31±8	7	84±5	NI	78±3	10±1	NI	99±1
A. nidulan	73±4	NI	42±8	NI	80±1	NI	47±1	69±6	35±6
A. terreus	60±3	60±8	50±4	45±2	10±1	55	80	33±6	84±7
A. ochraceus	NI	62±7	53±2	NI	45±1	61±10	100	68±8	96±4
A. versicolor	59±4	59±9	37±5	37±2	33±3	33±2	97±0	56±3	63±10
A. carbonarius	64±5	NI	43±7	52±4	36±7	52±3	47±1	79±6	100
A. ustus	33±3	NI	56±3	33±3	NI	67±5	100	78±5	99±1
A. glaucus	NI	69±5	24±8	62±5	NI	60±4	67±7	67±8	100

KEY:NI - No Inhibition

Moringa oleifera extract inhibited mycelia growth of all the *Aspergillus* species except *A. niger* aggregate, *A. fumigatus* and 3% inhibition of *A. foetidus* (Table 5). Inhibition of the isolates might be due to presence of alkaloids, flavonoids, phenols, saponins, steroids and tannins which can bind to the cytoplasmic membrane and cause membrane permeabilization [44,45]. Marrufo et al. [46] explained that at higher concentration of essential oil, *A. niger* was inhibited. Nkya et al. [47] reported that ethyl acetate extract of *M. oleifera* leaves had antifungal activities against *A. niger* which was in agreement with the findings of this study. The author added that, part of plant harvested for antifungal test and the extraction solvent could bring forth varying results.

Ganoderma lucidum has been reported to inhibit mycelia growth in fungi [48]. The fungus inhibited all the fungi species tested yielding varying percentages (Table 5). No fungal growths were recorded by *A. ustus* and *A. ochraceous* in plates incorporated with the extract. *Aspergillus* species tested all showed high percentages of inhibition though 10±1.09% inhibition was recorded by *A. fumigatus.* The results were in agreement with report of Rajech and Dhanasekaran [49]. Nithya et al. [50] report, using ethyl ether also agrees with the result presented in the study though at 16.8 mm inhibition. *G. lucidum* produces ganoderic acid, ganodermin, triterpenes and polysaccharides which inhibit mycelia growth [48].

Lippia alba contain geranial, neral, geranoil, and trans-β-caryophyllene confers antifungal properties on the plant extract [51]. Earlier report by the authors is at variance with the result presented in this study showing inhibition against *A. fumigatus*. At higher concentration and with a different solvent, inhibition of *A. fumigatus* may be feasible. Another author added that the antifungal properties of the plant results from limonene, bicyclosesquiphellandrene present in its essential oil; but Ara et al. [52] in their own submission, explained that the solvent system to some degree confers potency on *L. alba* against fungi isolates and other microorganisms.

4. CONCLUSION

The challenge of fungal contamination of agricultural food crops in Ondo State, Nigeria include losses recorded in the value and quantity of crops after harvesting, economic losses and quality time wastage for farmers. The study showed that plant parts contain potent antifungal phytochemicals that are able to inhibit fungal growth of *Aspergillus* species regarded as storage fungi. Finally, phytochemicals present in plant parts is majorly responsible for the plant antifungal activities hence further research work is necessary to isolate these novel chemicals and turn them into products that the farmers can utilize to check mold infestation and the resulting mycotoxin production more so that they are eco-friendly. The necessity to meet minimum standards of hygiene for food crop by exporting countries also makes the study very relevant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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