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Full Length Research Paper

Comparative antimicrobial activity of *Acacia nilotica* L. leaves extracts against pathogenic bacteria and fungi

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The comparative *in vitro* antimicrobial activity of ethanol and chloroform leaves extracts from *Acacia nilotica* L. was studied. Leaves extracts exhibited considerable bacteriostatic activity against two Grampositive and three Gram-negative strains. The antimicrobial action was compared with the effect of doxycycline antibiotic. The maximum zone of inhibition of 29 mm diameter was observed in *Escherichia coli* with ethanol extract while a minimum 8 mm zone of inhibition was found in *Bacillus subtilis*. *Klebsiella pneumoniae* showed marked resistance towards both ethanol and chloroform extracts. Among the fungal strains tested, *Aspergillus flavus* exhibited maximum sensitivity action of the extracts. This analysis revealed the high antimicrobial activity in the ethanol extract of *A. nilotica* L. than chloroform extract. It is recommended to isolate, identify and integrate the bioactive principle in these pathogens management.

Key words: Acacia nilotica L., ethanol, extract, chloroform, pathogenic, minimum inhibitory concentration (MIC).

INTRODUCTION

Medicinal plant researchers pursued with several goals like the development of low cost therapeutic compounds and the discovery of prototypic drugs (Elisabetsky, 1991). *Acacia nilotica* L. is the member of the family Mimosaceae and is known as babul in Pakistan. *Acacia* is a multipurpose nitrogen fixing tree legume. It occurs from sea level to over 2000 m and withstand extreme temperatures (> 50°C) and air dryness but sensitive to frost when it is young (Kiran and Bargali, 2009). It is widely spread in subtropical and tropical Africa from Egypt to Mauritania southwards to South Africa, and in Asia eastwards to Pakistan and India (Bennison and Paterson, 1994).

Phytochemical analysis of the aerial parts of the plant demonstrated the presence of polyphenolic compounds and flavonoids in the flowers. Tannins, volatile oils, glycosides, coumarins, carbohydrates and organic acids are reported in the fruits (El Shanawany, 1996). Babul has

*Corresponding author. E-mail: amjadqau2009@gmail.com. Tel: +923464318298. Author(s) agree that this article remain permanently open access under the terms of the <u>Creative Commons Attribution</u> <u>License 4.0 International License</u> been reported to contain l-arabinose, catechol, galactan, galactoaraban, galactose, N-acetyldjenkolic acid, N-acetyldjenkolic acid, sulphoxides pentosan, saponin and tannin. Seeds contain crude protein 18.6%, ether extract 4.4%, fiber 10.1%, nitrogen-free extract 61.2%, ash 5.7%, silica 0.44%, phosphorus 0.29% and calcium 0.90% of DM (Pande et al., 1981).

A. nilotica L. leaves are very digestible and have high levels of protein. The fruits are higher in aspartic and glutamic acid but lower in most other amino acids. The methionine was absent from the fruit of Australian materials but present in the seeds of African material (Fagg, 2001; Spies and March, 2004). Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Various medicinal plants have been used for years in daily life to treat disease all over the world. They have been used as a source of medicine. The widespread use of herbal remedies and healthcare preparations, such as those described in ancient texts. like the Vedas and the Bible, has been traced to the occurrence of natural products with medicinal properties. In fact, plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines (Farombi, 2003).

A. nilotica L. especially and other Acacia species are used in local traditional medicine by people as remedy for various disorders like cancers/tumors (of ear, eye or testicles) and indurations of liver and spleen, condylomas and excess flesh. It may also be used for colds, congestion, coughs, diarrhea, dysentery, fever, gallbladder, hemorrhage, hemorrhoids, leucorrhea, ophthalmia, sclerosis, smallpox and tuberculosis (Hartwell, 1971). Bark decoction is drunk for intestinal pains and diarrhea. The resin is mixed with orange-flower infusion for typhoid convalescence.

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are chiefly due to synthesized during secondary metabolism of the plant (Prusti et al., 2008).

There are several reports on the antimicrobial activity of different herbal extracts (Bonjar, 2004; de Boer, 2005; Islam et al., 2008). Many plants have been found to cure urinary tract infections, gastrointestinal disorders, respiratory diseases and cutaneous infections (Brantner, 1994; Somchit, 2003). Cytotoxic compounds have been isolated from the species of *Vismia* (Hussein, 2003). According to the World Health Organization (WHO), medicinal plants would be the best source for obtaining variety of drugs (Nair, 2006). These evidences contribute

to support and quantify the importance of screening natural products.

In Pakistan, huge varieties of medicinal plants are available (Dastur, 1970). Most of these plants are being used for therapeutic purposes without specific knowledge of their active ingredients. In fact, Pakistani medicinal plants, for the purpose of drug development, are one of the least investigated sources of natural compounds (Satyavati et al., 1976). The aim of the present study was to investigate the antibacterial and antifungal activity of the plant leaves ethanol and chloroform extracts of *A. nilotica* L. against both Gram-positive and Gram-negative bacteria and fungal. The anti-microbial activity of the plant leaves extracts was compared with that of standard antibiotic Dox (Doxycycline).

MATERIALS AND METHODS

All the experiments were done in three replicates and average values were used.

Anti-microial activity of Acacia nilotica L.

All the experimentation was done in aseptic area under laminar air-flow cabinet.

Anti-bacterial activity

Antibacterial activity of solvent extracts, ethanol and chloroform were determined by Well-diffusion method on nutrient agar medium according to Lino and Degracious (2006), with slight modifications.

Plant material and preparation of extract

A. nilotica L. species were collected from Quaid-i-Azam University, Islamabad, Pakistan. *A. nilotica* was identified and voucher specimens were deposited in the Herbarium, Department of Plant Sciences, Quaid-i-Azam University, Islamabad. Leaves of *A. nilotica* were rinsed with distilled water and kept under shade till drying. Leaves of the plants were weighed. Extraction from dried leaves was carried out by simple maceration process. The leaves were taken and grounded to coarse powder. The powder was suspended in 75% ethanol and 75% chloroform for 3 to 7 days at 60°C in extraction bottle. After two weeks, mixture was filtered twice, using Whatman-41 filter paper. Ethanol and chloroform was then completely evaporated by rotary evaporator to obtain the extract. Extracts were stored at 4°C for screening of anti-bacterial activity.

Preparation of samples

The extract (15 mg) was dissolved in 1 ml of dimethyl sulphoxide (DMSO). This stock solution 15 mg/ml was again diluted, thus 8 concentrations of the extract were prepared that is, 15, 12.50, 10, 7.5, 5.00, 3.00, 2.00 and 1.00 mg/ml. Along with these solutions of standard antibiotic, 2 mg/ml of the DOX was also prepared. The solutions of the extracts are used for test control. Standard antibiotics and pure DMSO were used for positive and negative

 Table 1. Different dilutions with DMSO (dimethyl sulphoxide).
 seeded with 1

 Petri plates we

Conc.	Stock Sol	DMSO	Final vol.		
(mg/ml)	(ml)	(ml)	(ml)		
15	1.00	0.00	1		
12.50	0.833	0.167	1		
10.00	0.66	0.334	1		
7.50	0.500	0.500	1		
5.00	0.334	0.666	1		
3.00	0.200	0.800	1		
2.00	0.133	0.867	1		
1.00	0.100	0.900	1		

control. Dilutions with DMSO are presented in Table 1.

Preparation of media for bacteria

Nutrient broth medium was prepared by dissolving 0.4 g/50 ml of distilled water for the growth of bacterial inoculum; pH was adjusted at 7.0 and was autoclaved. Nutrient agar medium was prepared by dissolving 2.3 g/100 ml of distilled water; pH was adjusted at 7.0 and was autoclaved at 121°C.

Mcfarland 0.5 barium sulphate turbidity standard

The standard was prepared by adding 0.5 ml 0.048 M barium chloride to 99.5 ml 0.36 N sulphuric acid. Barium sulphate turbidity standard (4 to 6 ml) was taken in screw capped test tube and poured to inoculums till the inoculum gave the same color as that of turbidity standard (Koneman, 1988).

Bacterial strains used

Five strains of bacteria were used in the study which was collected from Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan. Two were gram positive, *Staphylococcus aureus* (ATCC 6538) and *Bacillus subtilis* (ATCC 6633) and three were gram negative; *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 7221) and *Klebsiella pneumonia* (ATCC 10031). The organisms were maintained on nutrient agar medium at 4°C.

Preparation of inocula

Centrifuged pallets of bacteria from 24 h old culture in nutrient broth (SIGMA) of selected bacterial strains were mixed with physiological normal saline solution until a Mcfarland turbidity standard [10⁶ colony forming unit (CFU) ml⁻¹] was obtained. Then this inoculum was used for seeding the nutrient agar.

Preparation of seeded agar plates

Nutrient agar medium was prepared by suspending nutrient agar (MERCK) 2.3 g in 100 ml of distilled water; pH was adjusted at 7.0 and was autoclaved. It was allowed to cool up to 45°C. Then it was

seeded with 10 ml of prepared inocula to have 10⁶ CFU per ml. Petri plates were prepared by pouring 75 ml of seeded nutrient agar and allowed to solidify. Eight wells per plate were made with sterile cork borer (5 mm).

Pouring of test solutions: Incubation and measurement of zone of inhibitions

Using micropipette, 100 μ l of test solutions was poured in respective wells. These plates were incubated at 37°C. After 24 h of incubation, the diameter of the clear zones of inhibitions were measured by a ruler. Antibacterial activity of 8 dilutions of plant extract was determined against five bacterial strains.

Antifungal assay

The agar tube dilution method is used for antifungal activity of extract as reported by Choudhary et al. (1995).

Fungal strains used

Two fungal strains *Aspergillus niger* and *Aspergillus flavus* were used in this study. Each fungal strain was maintained on sabouraud dextrose agar medium at 4°C.

Media for antifungal assay

Sabouraud dextrose agar (MERCK) was used to grow fungus for inoculums preparation. Its composition was:

- A. Peptone complex 10 gm/L;
- B. Glucose 40 gm/L;
- C. Agar 15 gm/L.

Preparation of samples

The samples for antifungal assay were prepared from initial stock of 15 mg of extract of each sample per ml of DMSO. One sample of each extract was prepared, which were used for test. Slants without extract were used for negative control.

Assay procedure

Media for fungus was prepared by dissolving 6.5 gm/100 ml in distilled water pH was adjusted at 5.6. Test tubes were marked to 12 cm mark. The Sabouraud dextrose agar (MERCK) dispensed as 4 ml volume into screw capped tubes or cotton plugged test tubes and were autoclaved at 121°C for 21 min. Only single concentration 24 mg/ml was made. Tubes were allowed to cool to 50°C and nonsolidified SDA was loaded with 100 µl of 24 mg/ml plant extracts inserted by compound pipette from the stock solution. Tubes were then allowed to solidify in slanting position at room temperature. One slant of the extract sample was prepared for each fungus species. The tubes containing solidified media and test compound were inoculated with 4 mm diameter piece of inoculum taken from a seven days old culture of fungus. Negative control test tubes without extract were also inoculated. The test tubes were incubated at 28°C for 7 days. Cultures were examined twice weekly during the incubation. Reading was taken by measuring the linear length of

fungus in slant by measuring growth (mm) and growth inhibition was calculated with reference to negative control. Percentage (%) inhibition of fungal growth for each concentration of compound was determined by the following formula:



RESULTS

Antibacterial study of of Acacia nilotica L.

Extracts of ethanol and chloroform of *A. nilotica* L. were tested against five strains of bacteria. Two strains were Gram-positive that is, *S. aureus* and *B. subtilis* and three were Gram-negative that is, *E. coli, K. pneumoniae* and *P. aeruginosa*. Reading of inhibition zones were taken in millimeter (mm). All dilutions of extracts were made in DMSO. This solvent has no effect on the growth of bacteria. Eight dilutions of plants extracts sample was made that is, 15, 12.50,10, 7.5, 5.00, 3.00, 2.00 and 1.00 mg/ml. Only one concentration of 2 mg/ml of Doxycycline standard antibiotic was made and 100 ul of each plant extracts dilutions were introduced into the wells made by sterile cork.

The antibacterial activity of both ethanol and chloroform extracts from A. nilotica L. leaves against all test organisms was reduced with decrease of extracts concentrations. E. coli showed the highest inhibition zone (29 mm) with ethanol extract at the concentration of 15 mg/ml, while minimum inhibitory concentration (MIC) value was 1.00 mg/ml, MIC indicated 19 mm inhibitory zone. The inhibition zone reduced gradually with reduction of extract concentration to 25 mm at 12.5 mg/ml, 23 mm at 10 mg/ml, 22 mm at 7.5 mg/ml, 21 mm at 5 mg/ml, 20 mm at 3 mg/ml, 18 and 16 mm at concentrations of 2 and 1 mg/ml, respectively. The chloroform extract showed smaller inhibition zones, 18 mm at concentration 15 mg/ml and 15 mm at 12.5 mg/ml, 11 mm at 10 mg/ml, 08 mm at 7.5 mg/ml, 06 mm at 5 mg/ml, 05 and 04 mm at concentrations 3 and 2 mg/ml, respectively. The organism showed resistance towards chloroform extract at concentration of 1 mg/ml (Table 2 and 3). At 2 mg/ml, concentration of standard antibiotic doxycycline showed 38 mm inhibition zone.

K. pneumoniae showed marked resistance towards both ethanol and chloroform extracts at concentrations of 3, 2 and 1 mg/ml extracts. It was only affected by 15, 12.5, 10 and 7.5 mg/ml of the *A. nilotica* L. ethanol and chloroform extracts. Whereas MIC value was 1.00 mg/ml. MIC indicated 14 mm inhibition zone. Standard antibiotic doxycycline exhibited 25 mm inhibition zone at the concentration of 2 mg/ml. Only one concentration of antibiotic was made against test bacteria. On the other hand, *S. aureus* had a marked sensitivity towards both ethanol and chloroform extracts except with 2 and 1 mg/ml chloroform extracts. This sensitivity was markedly reduced with decrease in extract concentration.

B. subtilis tended to show the smallest inhibition zones at all concentrations of both ethanol and chloroform extracts when compared with other organisms. It also had a marked clear resistance against chloroform extracts at 3. 2 and 1 mg/ml concentrations. Here also MIC value was 1.00 mg/ml, and MIC exhibited 08 mm zone of inhibition. Standard antibiotic doxycycline showed 47 mm inhibition zone. The antimicrobial activity of ethanol and chloroform extracts from the A. nilotica fruits against P. aeruginosa was more effective than *B. subtilis*. All concentrations of ethanol and chloroform extracts showed inhibition zones except chloroform extract at concentrations of 3, 2 and 1 ma/ml. Whereas MIC value was 2.00 ma/ml. MIC showed 09 mm inhibition zone, whereas at 1.00 mg/ml extract did not show any antibacterial activity. Standard antibiotic doxycycline showed 14 mm inhibition zone. The ethanol and chloroform extracts from the A. nilotica gave smaller inhibition zones when compared with Dox antibiotic except fewer concentrations.

Antifungal study of Acacia nilotica plant

This study was done to check antifungal activity of A. nilotica L. plant. Only one concentration of plant extracts were prepared by dissolving 24 mg/ml in solvent dimethylsulfoxide. The fungi used in this study were A. niger and A. flavus. After inoculation and incubation of the samples for about one week, antifungal assay gave the following results. A. nilotica L. ethanolic extracts showed 4.91% and 116 mm growth inhibition while 7.0% and 93 mm growth inhibition against A. niger. A. nilotica L. ethanolic extracts showed 4.61% and 124 mm growth inhibition while 10.9% and 98 mm growth inhibition against A. flavus. These plant extracts were considered as test and control that is, with and without extract growth of fungus on media in the test tubes. The species A. niger gave 122, 100 mm growth in ethanolic and chloroform extracts and it was considered as control, whereas A. flavus gave 130, 110 mm growth in test tube and it was also taken as control. All antifungal results were compared with control in test growth and control was taken as standard to compare the growth of inhibition in test.

DISCUSSION

Plant based drugs are gaining popularity because of several advantages such as fewer side effect, better patient tolerance, relatively less expensive and acceptance

Bacteria	Inhibition zone (mm)			Ethanol extract (conc.)			Inhibition zone (mm)			Chloroform extract (conc.)						
	15	12.5	10	7.5	5	3	2	1	15	12.5	10	7.5	5	3	2	1
E. coli	29	25	23	22	21	20	18	16	18	15	10	08	06	05	04	0 [®]
S. aureus	24	22	21	19	17	15	12	11	14	10	08	06	05	04	0 [®]	0®
P. aeruginosa	16	14	13	12	11	10	10	09	11	10	08	06	04	0 [®]	0 [®]	0 [®]
K. pneumonia	21	20	19	17	14	0®	0®	0®	16	13	09	05	0®	0®	0®	0®
B. subtilis	15	14	13	12	11	10	09	08	11	10	08	05	04	0 [®]	0 [®]	0 [®]

 Table 2. Anti-bacterial activity of ethanol and chloroform extract from Acacia nilotica L. leaves.

Resistant.

 Table 3. Anti-microbial activity of standard antibiotic DOX (Doxycycline)

Destaria	Inhibition zone (mm) Dox (conc.)
Bacteria	2 mg/ml
E. coli	38
S. aureus	39
P. aeruginosa	14
K. Pneumonia	25
B. subtilis	47

due to a long history of use, especially herbal medicines provide rational means for the treatment of many diseases that are incurable in other system of medicine. The results of the present study provide a scientific validation for the popular use of the medicinal plants studied and serve as a guide which may help in selection of plants with antimicrobial activities for further phytochemical work on the isolation and the identification of the active compounds.

Pakistan is rich in diversity of plants. People living in rural areas are interested in the use of plant-based drugs, because plant based drugs have no side effects and they are inexpensive. *A. nilotica* L. appear to have potential for testing as a plant of high medicinal value for various antimicrobial activities as well as other biological activities. This plant is abundantly found in Pakistan and easily accessible.

The present study showed that *A. nilotica* L. leaves extracts were effective inhibitors of bacterial and fungal growth. The extracts of the plant showed varying degrees of activity against Gram-positive and Gram-negative bacteria as well as fungi. It showed inhibitory zones ranging from 4 to 29 mm against five strains of bacteria; whereas, at 3, 2 and 1.00 mg/ml against *K. pneumoniae* it did not show any antibacterial activity. Satish et al. (2008) found that aqueous extract of the *A. nioltica* L. exhibited significant study against the test bacteria. *A. nilotica* L. showed zones of inhibition from 9 to 35 mm, whereas in his study, zones of inhibitions were from 4 to 29 mm, and

the extracts was of ethanol and chloroform. The results revealed that ethanol extract was more effective against all test organisms than chloroform extract. This may be due to the ability of the ethanol to extract a wide range of chemical compounds of the plant leaves while the chloroform might have extracted less number of the constituents.

The leaves extracts showed higher activities against *E. coli* compared with other test bacteria (Gram negative). As *A. nilotica* L. leaves contains flavonoids, polyphenolic compounds, tannins, glycosides, organic acids and coumains (El-Shanawany, 1996), the anti-microbial activity of the plant leaves might be due to the polyphenolic compounds. It was found that the polyphenolic compounds are responsible for the anti-bacterial activity of plants.

The studies of Cheesbrough (1984) also indicated that polyphenolic compounds and/or volatile oils cause inhibition of a wide range of microorganisms. Phenol is well known as a chemical antiseptic. The presence of tannins may have accelerated wound healing probably due to their astringent effect. Al-Yahya et al. (1990) found that both ethanol and chloroform extracts from *A. nilotica* L. fruit were equally effective against both *B. subtilis* and *S. aureus* and that the ethanolic extracts was also active against *P. vulgaris*.

Saini et al. (2008) found that the mathanolic extracts of *A. nilotica* (pods) and *Acacia catechu* (bark) were reported to be most active against different bacterial and fungal strains. The methanolic extract of *A. nilotica* (pods) showed highest activity against *E. coli, S. aureus* and *A. niger.* In this study, *A. nilotica* L. showed highest activity against *E. coli followed by K. pneumoniae, B. subtilis* and *P. aeruginosa.* Plant was active against all test micro organism. Table 4 and Figure 1.

Banso (2009) using agar diffusion method found that the ethanolic stem bark extract of *A. nilotica* L. produced antimicrobial activity against *Streptococcus viridans*, *B. subtilis*, *S. aureus*, *E. coli* and *Shigella sonnei*. The extract contained the active principles– terpenoids, tannins, alkaloids, saponins and glycosides. MIC value against *B. subtilis* and *E. coli* was 35 and 45 mg/ml. In this study, it

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Fungi	Linear growth i	n control (LGC) (mm)	Linear growth	in test (LGT) (mm)	% inhibition		
	Ethanol	Chloroform	Ethanol	Chloroform	Ethanol	Chloroform	
A. niger	122	100	116	93	4.91	7.0	
A. flavis	130	110	124	98	4.61	10.9	

Table 4. Anti-fungal activity of ethanol and chloroform extracts from Acacia nilotica L., leaves



Figure 1. Antibacterial activity of Acacia nilotica L. against Escherichia coli (a), Pseudomonas aeruginosa (b) Klebsiella pneumonia (c) and Bacillus subtilis (d).

it was found that ethanolic extract of *A. nilotica* L. exhibited significant activity against all test pathogens. MIC value against *E. coli* and *B. subtilis* was 1 mg/ml. This is in contrast with MIC value of the work of Banso (2009).

Al-fatimi et al. (2007) found that the methanolic extract of *A. nilotica* L. did not show any activity against *E. coli* and *P. aeruginosa*, whereas it exhibited antimicrobial activity against *S. aureus*, *B. subtilis* and *M. flavus*. They found that it gave antifungal activity against *Candida krusei*, *A. fumigatus*, *Absidia corymbifera* and *Trichophyton mentagrophytes*. In this study, it was found that ethanolic and chloroform extracts of *A. nilotica* L.



Figure 2. Zones of inhibition (mm) showing Antibacterial activity of *Acacia nilotica* L., against *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Bacillus subtilis* (a) and Antibacterial activity of standard antibiotic DOX (doxycycline) using 2 mg/ml concentration (b).

was equally efficient against gram negative *E. coli* and *P. aeruginosa* as well as against fungi. Figure 2

Dabur et al. (2007) analyzed that water extracts of *A. nilotica, Justicia zylanica, Lantana camera* and *Saraca asoca* were found to be the most active against bacteria as well as fungal pathogens. The wells containing a concentration of 9.375 to 150.0 μ g/ml extracts of water and methanol inhibited the visible growth of all the bacterial species. Methanol extracts of *A. nilotica* L. and *J. zylanica* exhibited good activity in the range of 18.75 to 75.0 μ g/ml. Whereas in this research, ethanolic and chloroform extracts of *A. nilotica* L. was prepared and it gave remarkable activity against all pathogens.

From the studies, it is concluded that the traditional plants may represent new sources of anti-microbials with stable, biologically active components that can establish a scientific base for the use of plants in modern medicine. These local ethnomedical preparations and prescriptions of plant sources should be scientifically evaluated and then disseminated properly and the knowledge about the botanical preparation of traditional sources of medicinal plants can be extended for future investigation into the field of pharmacology, phytochemistry, ethnobotany and other biological actions for drug discovery (Gandhiraja et al., 2009).

Conclusion

Results indicate the potential of this plant for further work on isolation and characterization of the active principle responsible for antimicrobial activity and its exploitation as therapeutic agent. All Pakistani medicinally important floras should be tested against all pathogens in order to develop new and cheaper drugs using modern techniques like thin layered chromatography (TLC), high performance liquid chromatography (HPLC) and spectrophotometery. There is need for phytochemical as well as biological activities of plants in Pakistan. There is need for developing drugs from plants as microorganisms are becoming resistant to antibiotics thereby creating health problems.

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Conflict of Interests

The author(s) have not declared any conflict of interests.

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