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Analgesic and Anti-inflammatory Activities of the Ethylacetate Extract of *Mitracarpus villosus* Leaves in Rodents

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Authors' contributions

This work was carried out in equal collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the effects of the ethylacetate extract of *Mitracarpus villosus* leaves using various *in-vivo* models of pain, inflammation and pyrexia.
Study Design: This study was designed to investigate the possible analgesic, anti-inflammatory and anti-pyretic effects of ethylacetate extract of *Mitracarpus villosus* leaves in rodents.
Place and Duration of Study: Department of Pharmacology and Toxicology, National Institute for Pharmaceutical Research and Development, Idu Industrial Area, Abuja, Nigeria; between September 2013 and February 2014.
Methodology: The effects of the extract on centrally and peripherally mediated pain were investigated in albino mice and Wistar rats. The anti-nociceptive effects of the extract were tested

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on acetic acid-induced abdominal writhing, oro-facial formalin-induced pain in mice and carageenaan–induced hyperalgesia in rats. The effects of the extract on inflammation and body temperature were determined using formalin induced paw oedema and Baker's yeast induced pyrexia respectively.

Results: The extract at 100 – 400 mg/kg significantly ($P \leq 0.05$) and dose-dependently inhibited acetic acid-induced abdominal writhing, decreased the time of face rubbing induced by formalin and increased the withdrawal threshold of rat paws injected with carageenaan to induced hyperalgesia. At 400 mg/kg, paw thickness induced by formalin was significantly reduced when compared to control. The analgesic and anti-inflammatory effects of the extract are comparable to pentazocine and diclofenac. Hyperthermia induced by Baker's yeast was significantly reversed by the extract in a manner similar to paracetamol.

Conclusion: The results obtained suggest that the ethylacetate extract of *Mitracarpus villosus* leaves may contain biologically active principles with potential analgesic, anti-inflammatory and anti-pyretic effects; thus supporting its use as a phytomedicine and buttressing the need for the isolation and identification of the biologically active constituent(s) of this plant.

Keywords: Formalin; hyperalgesia; mitracarpus; orofacial; pyrexia; phytomedicine.

1. INTRODUCTION

Pain as defined by the International Association for the study of pain (ISAP) is an unpleasant sensory and emotional experience which can be associated with actual or potential tissue damage. Pain could play an adaptive role in the survival of an organism by protecting it from injury, or it may be maladaptive as an expression of the pathologic operation of the nervous system [1]. Pain is a wide spread clinical problem and a discomforting situation that results to frequent visits to the doctor's office or administration of analgesic preparations. Pain, inflammation and pyrexia are associated conditions: Inflammation is the response of living tissue to injury which involves complex processes of enzyme activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair and pyrexia may result as a secondary impact of tissue injury, inflammation, infection or other conditions. Pyrexia is the body's defense mechanism which creates an environment where infections agents or damaged tissue cells cannot survive [2]. Damaged tissues initiate the enhanced formation, activation or release of chemical mediators such as protons, kinins, prostanoids, histamine, and serotonin which activate sensory neurones in pain, produce swelling by vasodilatation in inflammatory conditions and increase the synthesis of prostaglandin E2 near hypothalamic area thereby triggering the hypothalamus to elevate body temperature [3]. NSAIDs which act by inhibition of cyclooxygenase enzymes with subsequent inhibition of prostaglandin synthesis are frequently administered in the management of pain and inflammation [4].

Several plants have been in use for many decades as food and medicines, and the claim that they present with less harmful side effects has made their use very popular in the communities where these plants occur; the pain alleviating properties of many plants are widely acclaimed among communities of many developing countries [5]. Analgesics used in orthodox medicines which include narcotics and non-narcotics have been associated with side effects [6-7]. The continuing search for efficacious medicines with limited debilitating adverse reactions profile puts medicinal plants as sources of medicines or lead compounds for the discovery and development of safer drugs [8] and several plants with analgesic actions demonstrate anti-inflammatory and anti-pyretic effects.

Mitracarpus villosus (S.w) D.C (family - Rubiaceae) is a widely occurring annual weed that has been attributed with therapeutic properties. In West African traditional medicine, the plant *Mitracarpus villosus* has been used for the management of pain related conditions including headaches and toothaches [9]. In a previous study [10] which screened several plants in Congo Brazaville, the potential analgesic property of this plant was recorded and [11] the anti-inflammatory effects of this plant had been indicated. This study however aims to provide additional information on anti-nociceptive, anti-inflammatory and anti-pyretic properties of the ethylacetate extract of *Mitracarpus villosus* as a step towards the documentation of the pharmacological profile of this medicinal plant.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant material was collected by Mal Tanko Garba in the month of September 2013, around Idu, Abuja, Nigeria. The plant was identified and authenticated by Mrs. Grace Ugbabe of the Department of Medicinal Plant Research and Traditional Medicine, National Institute for Pharmaceutical Research and Development (NIPRD) Idu, Abuja where a voucher specimen (NIPRD/H/6606) was prepared and deposited for future reference.

2.2 Preparation of the Extract

The leaves were cleaned of debris, air-dried and crushed using a mortar and pestle to obtain a coarse powder. 250 g of powdered plant material was subjected to soxhlet extraction using 2.5 L ethyl acetate. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator and the concentrate was evaporated to dryness on a water bath to give a semi-solid (the extract) with a mean yield of 0.9±0.3% w/w.

2.3 Animals

Wistar rats (120 – 145 g) and Swiss albino mice (25- 32 g) bred and maintained at the Animal Facility Centre of NIPRD were used in these studies. They were housed in standard polypropylene cages with saw dust as beddings, under ambient conditions and fed on standard rodent feed (CAPS Plc. Ibadan) with free access to water *ad libitum*. The experiments were carried out and the animals were handled according to the "principles of Laboratory animal care" (NIH publication 85-23, revised 1985) and the Institutional Animal Ethical committee guideline. Steps were taken to minimise the number of animals used.

2.4 Pharmacological Tests

The ethyl acetate extract of the leaves of *Mitracarpus villosus* was suspended in a vehicle consisting of 0.5% Tween 80 in Normal saline. All drugs and extract were freshly prepared and administered via the intraperitoneal route at 30 min before tests were carried out. The doses used in these experiments were derived from previous studies. The animals were weighed and randomly placed into 6 groups of 6 animals/group and treatment of animals were carried out as follows: Group 1 served as negative control with the animals receiving the vehicle (0.5% Tween 80 in Normal saline), groups 2 – 4 received 100, 200 and 400 mg/kg of extract respectively, while groups 5 and 6 were positive control and animal were administered diclofenac 10 mg/kg, Pentazocine 40 mg/kg or paracetamol 100 mg/kg. All observations were taken by trained personnel who were unaware of the treatment schedule.

2.4.1 Acetic acid-induced writhing test

In this method, the number of abdominal constriction induced by intraperitoneal administration of dilute acetic acid was counted according to the method of Koster et al, 1959 [12]. The extract was administered 30 min before administration of acetic acid (10 ml/kg of 0.60% v/v), 6 animals per group were tested. Parallel tests were carried out using the vehicle and diclofenac as reference. A reduction in the number of abdominal writhes between control (vehicle) animals and extract treated groups was regarded as analgesic activity.

2.4.2 Oro-facial formalin test

In this study, animals were brought to the test chamber 1 h prior to the experiments in order to allow for adaptation to the environment. 30 min after intraperitoneal administration of extract or standard drugs, 20 µl of 2.5 % formalin was injected into the upper lip of the mice using a 27-guage needle. The animals were placed in a plexiglass observation chamber with a mirror placed at an angle of 45° underneath the floor in order to allow an unobstructed view of the formalin injected site by the observer. The mice were observed in the box and the time spent face-rubbing the injected area with the fore or hind paws was recorded with a chronometer. The behavioural responses were observed immediately after formalin injection for a period of 5 min and at 15 min up until 30 min after injection. Two distinct nociception times are shown to be induced following formalin injection; the first 5 min represents phasic/neurogenic pain - early phase, while the second period 15 and 30 min represents tonic/inflammatory pain - late phase [13]. The time spent by the animal face rubbing the injected area with fore/hind paws was regarded as nociception [14] and a reduction in the time was considered as anti-nociception.

2.4.3 Carageenaan induced hyperalgesia

The method described by Lannitti et al. 2012 [15], was followed with modification. This was

assessed using the rat hind paw withdrawal thresholds in response to mechanical stimulation using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Thirty min after intraperitoneal administration of extract/drug, each rat was placed in a clear acrylic cubicle with a metal grid floor which allows access to the underside of their paws, and they were allowed to acclimatize for 30 min before tests were conducted. A mechanical stimulus was applied to the plantar surface of one hind paw by a stainless steel filament (0.5 mm in diameter) exerting a linearly increasing pressure. A cut-off force of 50 g was pre-set to prevent tissue damage. The force (g) at which paw withdrawal occurred was automatically recorded. Each rat paw withdrawal threshold was calculated as the average of three consecutive tests performed at 5-min intervals. This was regarded as the nociception threshold. Testing was carried out before (0 mins) and at 30, 60, 120 and 180 min after intraplanter injection of carageenaan [16].

2.4.4 Anti-inflammatory test

In this test, 20 µl of freshly prepared 2% formalin was injected into the sub-plantar region of the right hind paw of each mouse to induce oedema. The paw thickness of each animal was measured using Vernier calliper 30 min before and 30 min after extract/drug administration. The extract (100 - 400 mg/kg), vehicle (10 ml/kg) and standard (diclofenac 10 mg/kg) were administered 30 min before administration of sub plantar formalin; 6 animals per group were tested. The drug treatments were continued for 3 consecutive days and paw oedema measured 30 min after drug treatment on each day. Measurement of paw oedema was continued up to 6 days following formalin injection. The percentage inhibition of the oedema was calculated for each treatment group relative to control [17].

2.4.5 Anti-pyretic test

Basal rectal temperature of mice was measured by insertion of lubricated digital thermometer into the rectum of the animals 30 mins prior and 30 min after treatment. The temperature was subsequently monitored hourly over a period of 4h.

In another set of animals, the effect of the extract on Baker's yeast induced hyperthermia was investigated. This test followed the method described by Tomazetti et al. [18] with modification. Rectal temperature was measured then animals were injected with a pyrogenic dose (0.135 g/kg, i.p) of Baker's yeast. Rectal temperature was recorded after 4 h and the animals that showed an increase of > 0.5℃ of rectal temperature were selected and treated with vehicle, extract or paracetamol 100 mg/kg. The temperature was measured hourly up to 4 h post treatment.

2.5 Statistical Analysis

Data were analysed using ANOVA followed by Bonferroni's post hoc test. The results were expressed as mean values±standard error of mean (SEM). The statistical significance between the mean values was determined at P≤0.05.

3. RESULTS

3.1 Effects on Acetic Acid Induced Abdominal Writhing

In this study, the ethyl acetate extract of *Mitracarpus villosus* (100 - 400 mg/kg; i.p.) showed a significant (P<0.05) dose dependent decrease in the number of abdominal writhes induced by dilute acetic acid (Fig. 1).

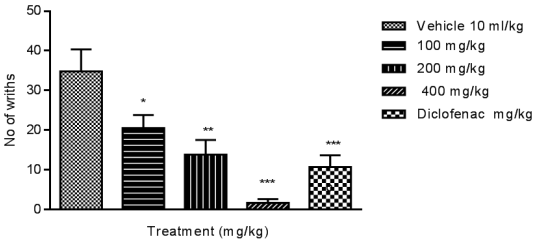

Fig. 1. Effect of the ethylacetate extract of *Mitracarpus villosus* on acetic acid-induced abdominal writhing in mice

Values are presented as mean±SEM (n = 6); P≤0.05 significant when compared to control

3.2 Effects on Oro-facial Formalin Test

The administration of extract of *Mitracarpus villosus* produced a decrease in the face rubbing behavioural response induced by formalin. The tested doses (100 – 400 mg/kg) produced a significant (P ≤ 0.05), dose dependent reduction in time in both early (phase 1) and late (phase 2) phases of pain when compared to control (vehicle). Pentazocine reduced the face rubbing time significantly in both phases. Paracetamol however produced significant change in the face rubbing period in phase one as represented in Fig. 2.

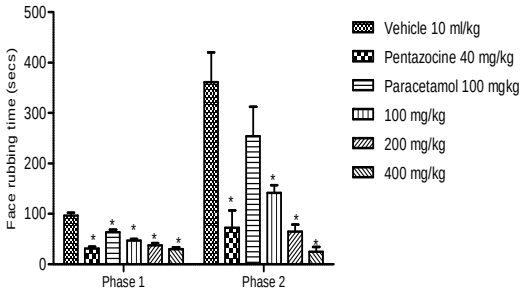

Fig. 2. Effect of the ethylacetate extract of *Mitracarpus villosus* on oro-facial formalin induced pain in mice

Values are presented as mean±SEM (n = 6) P≤0.05 significant when compared to control

3.3 Effects on Carageenaan Induced Hyperalgesia

Pre-administration of extract of *Mitracarpus villosus* significantly increased the mechanical withdrawal threshold of the hind paws compared to control. No statistical significant difference was detected between the groups treated with 100 and 200 mg/kg. At 400 mg/kg the withdrawal threshold was increased by 54.52% compared to the vehicle at 180 min (Fig. 3).

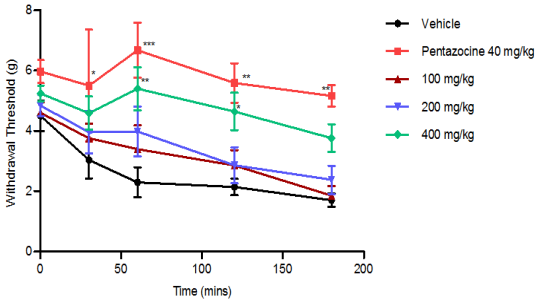

Fig. 3. Effect of the ethylacetate extract of *Mitracarpus villosus* on carageenaan induced hyperalgesia in rats

Values are presented as mean±SEM (n = 6); P≤0.05, significant when compared to control

3.4 Effects on Formalin Induced Paw Oedema

The results of the effect of ethylacetate extract of *Mitracarpus villosus* on formalin induced oedema is shown in Fig. 4. The extract produced a reduction of paw thickness which was significant at 400 mg/kg on day 3 of treatment.

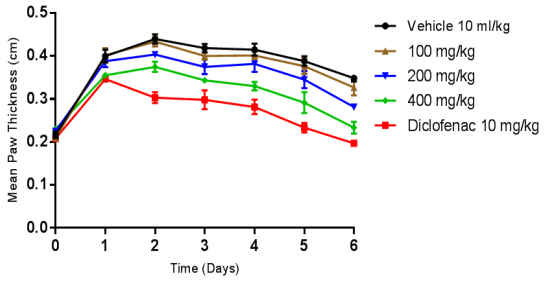

Fig. 4. Effect of the ethylacetate extract of *Mitracarpus villosus* on formalin induced paw oedema in mice

Values are presented as mean±SEM (n = 6); P≤0.05 significant when compared to control

3.5 Effect on Body Temperature

The extract caused a reduction in normal rectal body temperature up to 2 h after extract administration, but was reversed by 4h post treatment. Yeast caused an increase in body temperature 4 h after yeast administration, however this effect was significantly (P ≤ 0.05) reduced by the extract from 38.13±0.24 to 36.21±0.26℃ at 400 mg/kg within 4 h after extract administration. The effect was significant from 2 h post-treatment as shown in Fig. 5.

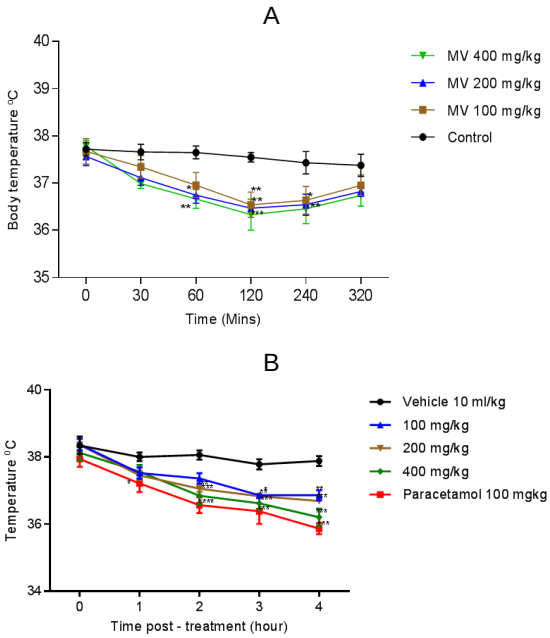

Fig. 5. Effect of *Mitracarpus villosus* extract on (A) normal body temperature in mice and (B) effect on Yeast induced pyrexia.

Values are presented as mean ± SEM (n = 6) P<0.05 significant when compared to control

4. DISCUSSION

The anti-nociceptive effect of the ethylacetate extract of *Mitracarpus villosus* leaves was investigated using various in-vivo models for both neurogenic and inflammatory pain. The extract significantly and dose dependently decreased the number of abdominal writhes induced by dilute acetic acid. The extract caused a reduction of the duration of face rubbing in the oro-facial formalin test while causing an increase of the withdrawal threshold in carageenaan induced hyperalgesia.

The acetic acid induced abdominal writhing is a visceral pain model that assesses peripheral anti-nociceptive activity [16]. Acetic acid causes inflammatory pain as a result of capillary permeability due to increase in level of endogenous substances in the peritoneum caused by the administration of the irritant [19]. The rising levels of these endogenous substances such as prostanoids, bradykinin, serotonin and histamine [20] produce localized inflammatory responses and stimulate pain nerve endings resulting in painful episodes. In this study, the extract decreased the number of abdominal writhes induced by dilute acetic acid. This result agrees with the study carried out by Makambila-Koubemba et al. 2011 [10]. The reduction exhibited by the extract is significantly different on comparison with control. The treatment with extract at 400 mg/kg produced an effect greater than the standard diclofenac at 25 mg/kg. This effect is similar to the action of the standard diclofenac, an analgesic agent with anti-inflammatory properties [21]. Pre-treatment with the extract decreased the pain sensation produced by acetic acid thereby suggesting anti-inflammatory mechanisms being involved in the analgesic action of *Mitracarpus villosus* given that the acetic acid method is a useful screening tool for anti-inflammatory analgesic agents [22].

Formalin test is a valid and reliable model of persistent clinical pain [23]. The oro-facial region is innervated by the trigeminal nerve [24]. This densely innervated area is the site frequently associated with headaches and pains relating to the teeth and related structures of the head, face and neck regions [25-26].The oro-facial formalin test exhibits two characteristic phases of pain. The early which denotes the neurogenic phase is due to direct activation of nociceptors and primary afferent fibres by formalin leading to release of bradykinin and tachykinins; this phase is inhibited by opioid analgesics [27]. The second phase/late phase is the inflammatory phase accompanied by the release of inflammatory mediators due to inflammatory reaction caused by tissue injury leading to release of histamine, serotonin, prostaglandin and excitatory amino acids [28]. This phase is associated with the activation of central sensitized neurons due to peripheral inflammation as well as increase in sensitivity/activity of primary afferents nociceptors at the injury site [29]. This phase is inhibited by NSAIDs and opioid analgesics [30]. The extract inhibited both phases 1 and 2 of pain in a manner similar to pentazocine a centrally acting analgesic [31] which also inhibited both phases of pain. Paracetamol in this study reduced face rubbing time in phase one and not in the second phase of pain even though it has been postulated that paracetamol affects both peripheral and central anti nociception processes [32]. The effect of the extract is greater than that produced by paracetamol at the doses tested. This observation may have been as a result of paracetamol possessing mild analgesic effect [33]. The significant reduction in the period of face rubbing by the extract in both early and late phases of the formalin test suggests that the extract of *Mitracarpus villosus* has activity in both central and peripherally mediated pain. The interaction of the extract of *Mitracarpus villosus* leaves with opioid receptors in the formalin test is a possible mechanism of its anti-nociceptive action. This test is used to investigate both peripheral and central mechanisms; centrally acting drugs such as opioids, inhibit both phases of pain equally.

Carageenaan induced hyperalgesia which exhibits a biphasic response is a method that assess inflammatory pain without any injury to the inflamed tissue; the first stage (0 - 2 h) is associated with the release of inflammatory mediators such as histamine, serotonin and kinins while the second phase (3h onward) is primarily due to the enhancement of inducible cyclo-oxygenase iso-enzyme, COX 2 and subsequently prostaglandins [34]. In this experiment the extract increased the withdrawal threshold of the paw of treated animals suggesting anti-nociceptive activity. It is possible that the anti-nociceptive effect demonstrated by the extract might have been as a result of the extract suppressing/reversing inflammatory processes by the inhibition of the inflammatory mediator substances [19] which have been implicated in the model of pain.

The formalin induced paw oedema is a model of sub-acute inflammation resulting from cell damage which provokes the production of

endogenous mediators that include histamine, serotonin, prostaglandins and bradykinin [35]. Formaldehyde induces inflammation by causing proliferation and migration of fibroblast which have the role of maintenance of the structural integrity of connective tissues; the ethylacetate extract of *Mitracarpus villosus* ameliorated inflammation caused by formalin thus indicating the plant maybe mediating anti-inflammatory activities by regulating the proliferation and migration of fibroblasts [36]. The standard diclofenac show significant reduction of paw oedema when compared with control values. This result agrees with an earlier study carried out by Ekpendu et al. 1994 [11]. It is also possible that this effect produced by the extract could also have been due to inhibition of COX-2. Prostaglandins play a significant role in the generation of the inflammatory response where they contribute to the development of the signs of acute inflammation [37]. Prostaglandin synthesis via COX-2 is vital in pain transmission; therefore the inhibition of COX-2 leads to pain relief [38]. Phytoconstituents such as flavonoids, terpenoids and tannins detected in *Mitracarpus villosus* have been reported to possess anti-nociceptive properties in other plants [4]. They are able to inhibit the inducible isoforms of cyclo-oxygenase (COX-2) as well as other mediators of the inflammatory process [39]. The terpenoid compound ursolic acid has been shown to possess analgesic and anti-inflammatory effects in another species of *Mitracarpus* [40] and other plants [41-42]. The presence of this compound in *Mitracarpus villosus* [43] may be contributing to the observed analgesic effect.

The extract caused a reduction in normal rectal body temperature up to 2 h after extract administration, but was reversed by 4 h post treatment. Yeast caused an increase in body temperature 4 h after yeast administration, however this effect was significantly ($P < 0.05$) reduced by the extract from 38.13±0.24 to 36.21±0.26℃ at 400 mg/kg within 4 h after extract administration. The effect was significant from 2 h post treatment.

Fever is associated with increase in production of the endogenous substances that include the potent pyretic agent PGE_2 in the hypothalamus; antipyretic activity is characteristic of compounds which have inhibitory effect on prostaglandin synthesis, it is therefore suggested that the anti-pyrectic activity exhibited by the extract which is similar to paracetamol may be by inhibition of cyclooxygenase and consequently prostaglandins. Flavonoids and terpenes have also been reported to possess anti-pyretic activity. This property may contribute to the observed effect [44]. In an earlier study, the ethylacetate extract of *Mitracarpus villosus* was shown to exhibit sedative actions possibly by modulation of the action of the CNS inhibitory neurotransmitter GABA [45]. Systemic administration of GABA agonists cause decrease in core body temperature elicited by possible action by that substance on the GABA-ergic system [46] and stimulation of GABA-ergic receptor systems have been shown to diminish the response to painful stimuli, as a result of which GABA agonists possess anti-nociceptive activity [47].

5. CONCLUSION

In conclusion, this study demonstrates that the extract of *Mitracarpus villosus* possesses anti-nociceptive effects against inflammatory and non-inflammatory mediated nociception, which validates its use as an herbal medicine thus supporting the isolation and identification of the biologically active constituents of the plant as anti-inflammatory analgesic agent.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national and Institutional laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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