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Effects of Hesperidin and Nepitrin (*Salvia rosmarinus*) on the Response of $GABA_A$ Receptors Expressed in *Xenopus* Oocytes and their Neuropharmacological Activities

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Salvia rosmarinus, previously known as *Rosmarinus officinalis*, has intense pleasant smell reminiscent of pine wood. *S. rosmarinus* has been widely used in traditional medicines and has long been known as the herb of remembrance. However, few studies have investigated the effects of non-volatile components of rosemary on central nervous system function. In this study, Bio-assay guided fractionation of the butanolic extract of *S. rosmarinus* led to the isolation of two compounds hesperidin and nepitrin. Hesperidin and nepitrin were evaluated on recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes. Hesperidin and nepitrin were found to be

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flumazenil insensitive negative allosteric modulators of high concentrations of GABA at $\alpha_1\beta_{2'}\gamma_{2L}$ $GABA_A$ receptors. Hesperidin and nepitrin allosterically inhibit the response of GABA at $\alpha_1\beta_{2'}\gamma_{2L}$ GABA receptors via a site other than the high-affinity benzodiazepine biding site.

Keywords: Salvia rosmarinus, hesperidin; nepitrin; $GABA_A$; neuropharmacological activities; allosteric Modulation.

1. INTRODUCTION

"γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian central nervous system (CNS) activating neurons through several pharmacologically and structurally different receptor subtypes" [1]. "GABA plays a crucial role in the excitatory: inhibitory balance in neuronal networks by inhibiting glutamatergic pyramidal neurons" [2,3]. $GABA_A$ receptors are chloride channels that can be activated by GABA and modulated by other several drugs, [4] which are composed of pentameric channels formed by the combination of three distinct subunits, according to the following stoichiometry: 2α:2β:1γ. "The $GABA_A$ receptor is possibly the most complex member of the nicotinoid superfamily containing five major ligand binding sites for GABA, benzodiazepines, barbiturates, picrotoxin, and neurosteroids" [5,6]. "The additional binding sites serve to allosterically modulate the response of the receptor to GABA. Allosteric modulators have no direct action on the $GABA_A$ receptor. GABA also activates $GABA_B$ receptors, which are widely distributed throughout the mammalian brain and spinal cord" [7]. "$GABA_B$ receptors are G-protein-coupled receptors (GPCRs) that function as heterodimers of $GABA_{B1}$ and $GABA_{B2}$ subunits" [8,9].

"Salvia Rosmarinus, (previously known as *Rosmarinus officinalis*), belongs to Lamiaceae family. Lot of studies of different species of Lamiaceae family and their effects on memory, anxiety, depression, and sleep disorders" [10]. *"S. rosmarinus* intense pleasant smell reminiscent of pine wood. *S. rosmarinus* ,traditionally known as rosemary, it is important medicinal plant which grows all over the world specially in most Mediterranean countries" [11,12]. "It is often cultivated for its aromatic oil. *S. rosmarinus* has a long history of being used in traditional medicine and its antioxidant properties are well known" [13-15]. *S. rosmarinus* has the capability to release the symptoms caused by respirational disorders, to stimulate hair growth, to reduce stress and mental alertness, and to treat Rheumatoid disease. Moreover it has a long history of being a herb of remembrance [16] and it is believed that memory is enhanced by the use of this plant [17,18]. "An extract of *S. rosmarinus* was found to enhance the production of the nerve growth factor (NGF), a protein vital for the growth and functional maintenance of nerve tissue" [19]. "Carnosol, a major constituent of *S. rosmarinus*, significantly increased the amount of tyrosine hydroxylase indicating that it may be effective for the treatment for Parkinson's disease (PD)" [20]. "The aerial parts of *S. rosmarinus* have been shown to possess antinociceptive activity" [21]. "Thujone, constituent of the essential oil of *S. rosmarinus*, was found to modulate $GABA_A$ receptor" [22]. Rosmarinic acid proved to have antidepressive activity and significantly reduces the duration of response in the forced swimming test and decreases the defensive freezing behaviour of mice exposed to conditioned fear stress [23,24], and increased the number of entries in the open arms during plus-maze task, suggesting an anxiolytic-like activity in rat [25]. "Isolated compounds from *S. rosmarinus* have shown to exert biphasic modulation of $GABA_A$ receptors, demonstrated CNS activity in mouse models of antinociception, antidepressant and anxiolysis" [26]. "The action of salvigenin, rosmanol, and hispidulin acted as positive modulators when applied in the presence of low concentrations of GABA but in the presence of high concentrations of GABA acted as negative modulators, demonstrating a biphasic action" [27]. "Nepitrin, isolated from *S. rosmarinus*, was evaluated for its memory enhancing effects. Nepitrin was found to dose-dependently inhibit AChE and BuChE enzymes. The observed effect was comparable to donezepil (AChE inhibitor) suggesting that nepitrin may have a mechanism of action similar to that of donezepil, which was supported by molecular docking studies" [28]. "*In vivo* studies for the memory enhancing effect of nepitrin was also demonstrated. In the Y-maze task nepitrin was found to reverse scopolamine induced amnesia and increase the discrimination index in the novel object recognition test NORT" [29]. "The antinociceptive activity of hesperidin was assessed using the pain induced functional impairment (PIFIR) model in rats and capsaicin-

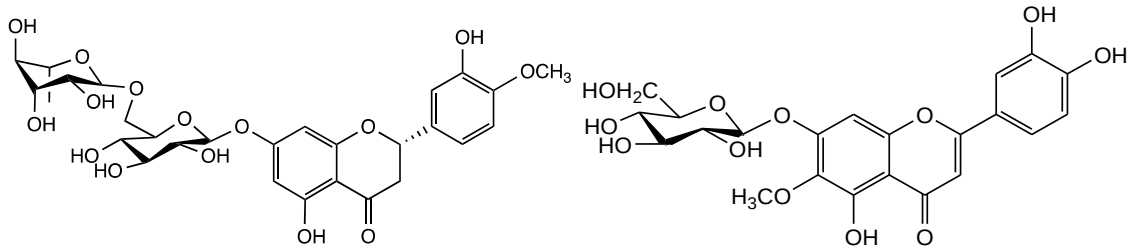

Fig. 1. Chemical structures of hesperidin and neptrin

induced nociception in mice. Hesperidin was found to exhibit a dose dependent antinociceptive activity. The antinociceptive effect produced by hesperidin in the PIFIR model was reduced by 36% with capsazepine pretreatment (TRPV1 selective antagonist) suggesting the involvement of the vanilloid (TRPV1) receptors" [30]. The current study reports the effects of the non-volatile constituents' hesperidin and neptrin (Fig. 1) on recombinant $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors expressed in *Xenopus laevis* oocytes.

2. MATERIALS AND METHODS

2.1 Plant Materials

The dried *S. rosmarinus* plants were purchased from herbal markets located in Amman. The plants were identified by Prof. Dawud AL-Eisawi (Department of Biological Sciences, Faculty of Science, University of Jordan). Voucher specimens were deposited at the herbarium of the University of Jordan.

2.2 Chemicals, Materials, Instrumentation and Drugs

All chemicals used were purchased from Aldrich Chemical Co. Ltd (St Louis, MO, USA) and were of highest commercially available purity. Silica gel for column chromatography (CC) was performed on silica gel (Merck silica gel 60H, particle size 5 - 40 µm) and Sephadex LH-20 gel. Thin layer chromatography (TLC) was performed on Merck aluminium backed plates, pre-coated with silica (0.2 mm, 60F254). UV-Spectra were recorded on Hitachi U-2000 double beam UV/Vis Spectrophotometer. Mass spectra were carried out on a Thermo Finnigan (Waltham, MA, USA) PolarisQ Ion Trap system using a direct exposure probe. Nuclear magnetic resonance ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively on a Varian Gemini spectrometer (Palo Alto CA, USA). Melting points were determined using a Stuart (Stone, Staffordshire, UK) SMP10 melting point apparatus.

2.3 Preparation of the Extracts and Solvent Fractionation

"The plant was dried and ground into fine powder (5 Kg). Defatting was done by extraction with petroleum ether (40 L) at room temperature for 7 days. The residual materials were then extracted by ethanol (3 times, 10 days each, 50 L) at room temperature to isolate the secondary metabolites. The combined crude ethanolic extracts were evaporated under vacuum. The resulting crude extract was partitioned between chloroform and water (1:1 v/v). The less polar organic compounds were extracted from water by *n*-butanol to give the butanol extract" [29].

2.4 Isolation of Constituents

"The butanol extract of *S. rosmarinus* (10 g) was adsorbed on 20 g silica gel and subjected to column chromatography (Φ28 × 4.5 cm). The columns were packed in chloroform and the polarity increased gradually by using methanol, until pure methanol was added resulting in four fractions (RVI-IX) according to their TLC fingerprint. Each fraction was further purified by a combination of column chromatography and preparative thin layer chromatography using suitable solvent systems" [29].

Yellow powder precipitated during treatment of fraction RVI with methanol. Then this powder was further washed with water to yield 60 mg of hesperidin (hesperetin-7-O-rutinoside (-Glc6-Rha)) as a pure yellow powder. Fraction RVII was washed with methanol to yield a yellow precipitate. Further washing of this solid with water afforded nepitrin (nepitrin-7-*O*-glucoside) which was soluble in the water layer.

2.5 Physical and spectroscopic data of the isolated compounds

Hesperidin**:** yellow powder; Melting point (m.p): 252-257 ˚C; UV λ_{max} (MeOH) nm: 332 (Band I),

283 (Band II); +NaOMe, 367, (Band I), 285 (Band II); +AlCl3, 353 (Band I), 285 (Band II); +HCl, 332 (Band I), 283 (Band II); ESMS m/z (%): 609 $[M-H]^-$ (100), 301.0 $[M-(Rha-Glc)+H]^+$ (14), 431.1 (7); ^{1}H-NMR (DMSO-d_6) δ ppm:12.0(1H, s, 5-OH),6.92 (1H, d, *J*=4.4 Hz, H-5'), 6.90 (1H, dd, *J*=9.8, 2 Hz, H-6'), 6.87 (1H, d, *J*=2 Hz, H-2'), 6.12 (1H, d, *J*=2.4 Hz, H-8),6.10 (1H, d, *J*=2.4 Hz, H-6),5.48 (1H, dd, *J*=12.4, 3 Hz, H-2),4.95 (1H, d, *J*=7.6 Hz, H-1''),4.50 (1H, s, H-1'''), 3.76 (3H, s, 4'-OMe),3.24 (1H, dd, *J*=13.2, 4 Hz, H-3β), 2.75 (1H, dd, *J*=17.2, 3.2 Hz, H-3α), 1.06 (3H, d, *J*=6 Hz, H-6'''); ^{13}C-NMR (DMSO-d_6) δ ppm: 197.5 (C-4), 165.6 (C-7), 163.5 (C-5), 162.9 (C-9), 148.4 (C-4'), 146.9 (C-3'), 131.3 (C-1'), 118.4 (C-6'), 114.6 (C-2'), 112.5 (C-5'), 103.7 (C-10), 101.0 (C-1'''), 99.9 (C-1''), 96.8 (C-6), 96.0 (C-8), 78.8 (C-2), 76.7 (C-5''), 76.0 (C-3''), 73.4 (C-4'''), 72.5 (C-2''), 71.1 (C-4''), 70.7 (C-3'''), 70.0 (C-2'''), 68.8 (C-5'''), 66.5 (C-6''), 56.1 (4'-OMe), 42.5 (C-3), 18.3 (C-6''').

***Nepitrin*:** yellow powder; ESMS m/z (%): 476.8 $[M-H]^+$ (100), 314.9 (7), 299.7 $[M-(Glc)+H]^+$ (9); ^{1}H-NMR (DMSO-d_6) δ ppm: 8.02 (1H, d, *J*=8.8 Hz, H-5'), 7.51 (1H, d, *J*=2 Hz,H-2'), 7.48 (1H, dd, *J*=7, 1.8 Hz, H-6'), 7.08 (1H, s, H-3), 6.80 (1H, s, H-8), 4.95 (1H, d, *J*=7.6 Hz, H-1''), 3.83 (3H, s, 6-OMe); ^{13}C-NMR (DMSO-d_6) δ ppm: 182.6 (C-4), 165.0 (C-2), 156.9 (C-9), 152.9 (C-7), 152.6 (C-5), 150.6 (C-4'), 146.3 (C-3'), 132.9 (C-6), 121.8 (C-1'), 119.6 (C-6'), 116.4 (C-5'), 113.9 (C-2'), 106.1 (C-10), 103.1 (C-3), 100.6 (C-1''), 94.7 (C-8), 77.7 (C-5''), 77.1 (C-4''), 73.6 (C-2''), 70.0 (C-3''), 61.1 (C-6''), 60.7 (6-OMe).

2.6 Pharmacological Analysis

"Electrophysiological evaluation of the final extract and the isolated compounds from *S. rosmarinus* was carried out on functional assays using two-electrode voltage clamp methods on recombinant GABA receptors expressed in *Xenopus laevis* oocytes using the methods described previously" [31].

2.7 Drugs and Chemicals

Diazepam was kindly donated by the Department of Pharmacy, University of Peshawar. Imipramine, DMSO, Tween solution (TWEEN® 80, Sigma-Aldrich Co. LLC, USA), methanol (Merck, Germany) and tramadol hydrochloride and flumazenil (98%, Sigma-Aldrich, USA) and pentylenetetrazol (Tokyo chemical industries Co Ltd) were purchased for the study. All chemicals and solvents used in this research were of analytical grade.

3. RESULTS

3.1 Electrophysiology

Hesperidin produced no effect on sham-injected oocytes or at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors when administered alone. "Hesperidin inhibited currents due to 100 µM GABA with an IC_{50} of 42.95 µM (95% CI: 19.62 to 93.98) and a Hill coefficient of 1.08 ± 0.95 (Fig. 2). Hesperidin was not affected by the addition of 10 µM flumazenil neither at high nor at low concentrations of GABA. The maximum concentration of hesperidin applied was 100 µM due to its solubility" [27].

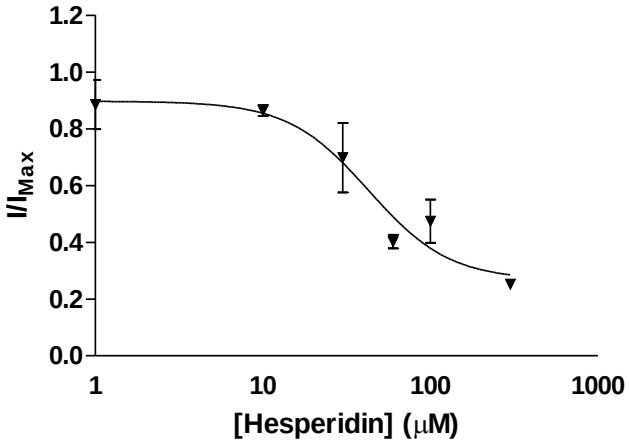

Fig. 2. Effect of hesperidin in the presence of GABA (100 µM) at $\alpha_1\beta_2\gamma_{2L}GABA_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n=3–6 oocytes)

GABA dose response curves were carried out both without and with hesperidin (100 µM) at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors (Fig. 3) with an EC_{50} of 782 µM (95% CI: 29.37 to 20834) and a Hill coefficient of 2.89 ± 0.71.

Nepitrin produced no effect on sham-injected oocytes or at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors when administered alone but inhibited currents due to 100 µM GABA with an IC_{50} of 98.7 µM (95% CI: 64.41 to 151.4) and a Hill coefficient of 2.12 ± 0.77 (Fig. 4) and shifted the GABA dose response curves at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors to the right, increasing the mean GABA EC_{50} from 123.3 to 193.6 µM (95% CI: 141.4 to 265.1), with a Hill slope of 1.05 ± 0.15 (compared to 0.73 ± 0.07 in the case of GABA alone) (Fig. 5). Nepitrin was not affected by the addition of 10 µM flumazenil neither at high nor at low concentrations of GABA.

EC_{50} and IC_{50} values were unable to be calculated for most of the compounds investigated due solubility limits. However, for comparison Table 1 summarizes the effect of 100 µM of each compound on the maximal response and EC_5 GABA response.

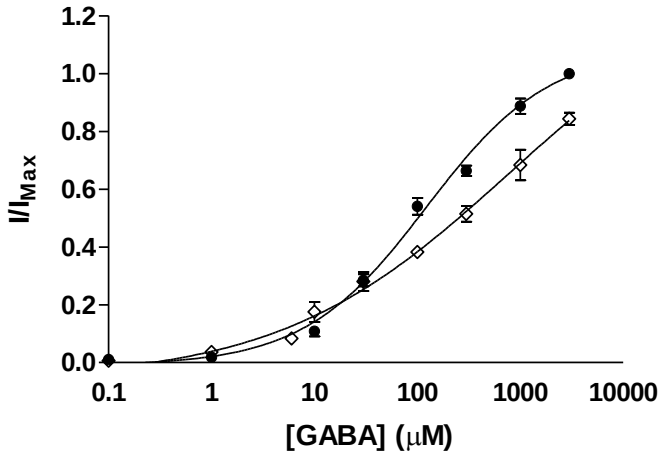

Fig. 3. Dose response curves of GABA (●) and GABA in the presence of 100 µM (◊) hesperidin at human $\alpha_1\beta_2\gamma_{2L}GABA_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n=3–6 oocytes)

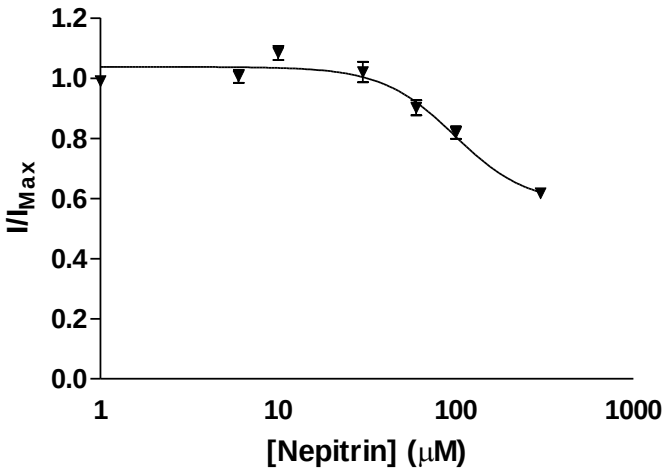

Fig. 4. Effect of nepitrin in the presence of GABA (100 µM) at $\alpha_1\beta_2\gamma_{2L}GABA_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n=3–6 oocytes)

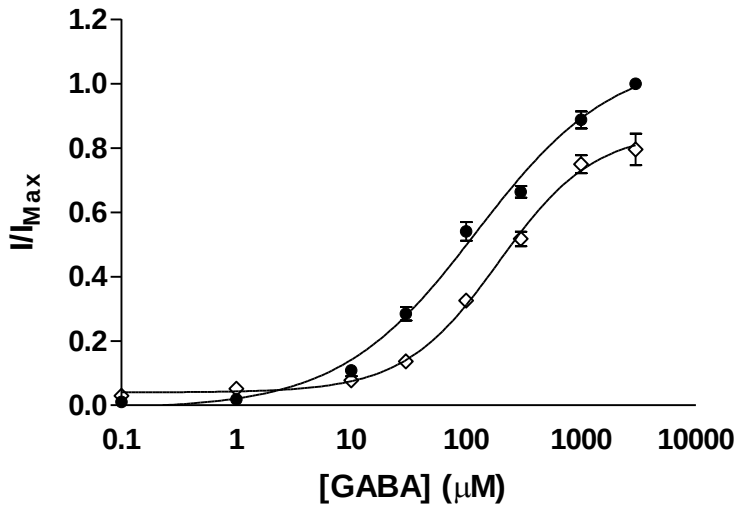

Fig. 5. Dose response curves of GABA (●) and GABA in the presence of 100 μM (◊) nepitrin at human $\alpha_1\beta_2\gamma_{2L}$GABA$_A$ receptors expressed in *Xenopus* oocytes. Data are the mean ± SEM (n=3–6 oocytes)

Table 1. Percentage inhibition and enhancement values for the isolated compounds

Compound	% Inhibition[a]	% Enhancement[b]
Hesperidin	52.6	-
Nepitrin	18.3	-

[a] *percentage inhibition of maximal GABA response by 100 μM compound*
[b] *percentage enhancement of GABA EC$_5$ response by 100 μM compound*

4. DISCUSSION

Hesperidin has previously been found to have sedative and sleep enhancing properties and when injected concurrently with diazepam the sedative effect is greatly increased [32-34]. "However, hesperidin did not potentiate the response of $\alpha1\beta2\gamma2$ GABA$_A$ receptors to low doses of GABA, supporting the suggestion that the sedative effects of hesperidin are not mediated via GABA receptors" [32]. "Systemic administration of hesperidin produced a significant reduction in the phosphorylation state of extracellular signal-regulated kinases 1/2 (pERK 1/2) in the cerebral cortex, cerebellum and hippocampus. However, no effect on pERK 1/2 was found for neohesperidin which lacks sedative properties" [35]. A study by Loscalzo *et al.* has also suggested "the involvement of opioid receptors in the sedative and antinociceptive effects of hesperidin" [36]. At high doses of GABA (100 μM) hesperidin inhibited the GABA response with an IC$_{50}$ of 42.95 μM. GABA dose response curves were carried out both without and with hesperidin (100 μM), at $\alpha1\beta2\gamma2$LGABA$_A$ receptors hesperidin shifted the GABA dose response curve to the right with an EC$_{50}$ of 782 μM. The effect of hesperidin was not altered by the addition flumazenil either at high or low concentrations of GABA indicating that actions of hesperidin are not mediated via high-affinity benzodiazepine sites. This is consistent with the finding that hesperidin was unable to modify [3H]-flunitrazepam binding to rat cerebral cortex synaptosomal membranes [32].

Nepitrin has been reported to exert anti-pyretic and weak analgesic activity [37]. To date, no studies have investigated the action of nepitrin at GABA$_A$ receptors. Nepitrin inhibited currents due to 100 μM GABA with an IC$_{50}$ of 98.7 μM and shifted the GABA dose response curves at $\alpha1\beta2\gamma2L$ GABA$_A$ receptors to the right, with an EC$_{50}$ of 193.6 μM, indicating that nepitrin acts as a negative modulator at this receptor, and is more potent than hesperidin. The action of nepitrin was not affected by the addition of flumazenil at either high or at low concentrations of GABA indicating that nepitrin is does not act at the 'high-affinity' benzodiazepine binding site.

Both hesperidin and nepitrin were found to be insensitive to flumazenil at both high and low concentrations of GABA.

5. CONCLUSIONS

Salvia rosmarinus (rosemary) has a long and pan-cultural history as traditional treatments for cognitive deficits, with the medicinal use of this herb as cognitive enhancers developing indepently in different cultures. $GABA_A$ receptors are known to be implicated in memory and cognition with enhancers of GABA function such as benzodiazepines having a well-documented detrimental effect on cognitive function. This study has focused on the effect of hesperidin and nepitrin at $\alpha_1\beta_2\gamma_{2L}$ $GABA_A$ receptors, which are the dominant sub-type of GABA receptor in the central nervous system. Both hesperidin and nepitrin were found to be insensitive to flumazenil at both high and low concentrations of GABA.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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