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Antioxidant, Antiproliferative Properties and Chemical Composition of the Ethanolic Extract from Leaves and Stems of Lebanese *Anacyclus nigellifolius* Boiss

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Authors' contributions

This work was carried out in collaboration between all authors. Author AH was the responsible of the study. Authors AS and HR wrote the protocol and wrote the first draft of the manuscript. Author FAS performed the statistical analysis. Authors SZ and MN managed the literature searches and the analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Objective: The present study aimed to determine the content of the ethanolic extract from leaves and stems of fresh Lebanese *Anacyclus nigellifolius* Boiss in primary and secondary metabolites and to evaluate its antioxidant and anti-tumor activities on Human lung A549 adenocarcinoma cells. Also, the effect of low doses of chemotherapeutic agent the Cisplatin (CDDP) combined with *A. nigellifolius* on these cell lines has been studied.
Methods: Phytochemical screening of the extract was done using standard classical tests. Total phenolic and total flavonoid contents have been determined using the Folin–Ciocalteau reagent

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and aluminum chloride method respectively. Also, different classic methods have been used to quantify alkaloids, lipids and ash. On the other hand the antioxidant ability has been estimated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method. In addition, the anti-tumor activity of the extract has been evaluated using Neutral Red assay.

Results: The obtained results of the phytochemical screening showed the existence of terpenoids, flavonoids, phenols, saponin, glycosides and tannins in ethanol extract. In addition, this extract has showed higher contents in both total phenolic and total flavonoids. On the other hand, the DPPH method showed strong scavenging activity with an IC_{50}=0.178 mg/mL. Moreover, an antiproliferative activity was observed by inhibiting the viability of A549 cell line in a dose-dependent manner.

Conclusion: All these results indicated that *A. nigellifolius* might be used in the prevention from some diseases related to the oxidative stress.

Keywords: Anacyclus nigellifolius; DPPH; antioxidant; anti-tumor; A549 cells.

1. INTRODUCTION

Cancer is a major public health problem in the United States and many other parts of the world. There are over 100 different types of cancer. Most cancers are named according to the organ or type of cell in which they begin. Lung cancer is one of the most important cancers. It is the leading cause of cancer death in men and women in the United States and represents about 28% of the total number of cancer deaths in 2010, despite it only forms 15% of new cases cancer [1]. In the 20th century the incidence and mortality of lung cancer has increased so dramatically that it can be considered one of the major epidemics of the former century [2]. Lung cancer is commonly classified into two main types that are NSCLC [Non-Small cell lung cancer] accounts for approximately 80-85% and SCLC [Small-cell lung cancer] represents 15-20% [3]. NSCLC comes from alveolar cells [4]. The term NSCLC is generally applied to the various types of bronchogenic carcinomas (those arising from the lining of the bronchi), which include adenocarcinoma, squamous cell carcinoma, and large cell undifferentiated carcinoma [5]. Adenocarcinomas are composed of bronchial basal cells and type II pneumocytes that appear in the periphery of the lung, and squamous comes from bronchial epithelial cells more centrally located [6]. The SCLC is derived from neuroendocrine cells " Kulchitsky cell " lung [7]. These cells are responsible for the secretion of the polypeptide hormones and are characterized by dense granules neurosecretory [8].

Environmental conditions are giving rise to a variety of free radicals leading to severe oxidative damage to proteins, lipids, enzymes and lipid peroxidation, with subsequent tissue injury. Natural antioxidant agents have an ability to scavenge free radicals implicated in the development of a number of disorders, including cancer, neurodegeneration and inflammation [9,10], giving rise to studies of antioxidants for the prevention and treatment of diseases.

It is well known that plants contain components of therapeutic value that may be used as alternative remedies for many diseases. Medicinal herbs are very interesting over the world for their antioxidant properties and their ability to act as efficient free radical scavengers [11,12].

Anacyclus nigellifolius Boiss belongs to the Asteraceae family. It is found in Lebanon, Syria and Turkey. It was used in the treatment of neuralgic and rheumatic affection of the head, face and teeth.

In this present work, we investigated for the first time, firstly the chemical composition of the ethanolic extract from fresh stems and leaves of Lebanese *A. nigellifolius*. Secondly, we evaluated the antioxidant activity of this extract using an *in vitro* method, the DPPH. Finally, we studied the effect of low doses of chemotherapeutic agent the Cisplatin (CDDP) combined with *A. nigellifolius* on human lung adenocarcinoma A549 cell line.

2. MATERIALS AND METHODS

All the used chemicals were purchased from Sigma-Aldrich chemicals co. All the solvents used in this study are analytical grade solvents.

2.1 Plant Collection and Preparation of Powders

A. nigellifolius was gathered from the south region of Lebanon at 300 m of altitude. The

biological authentication was carried out by the Professor George Tohme, the president of the Lebanese C.N.R.S. Leaves and stems have been separated from the plant and were well washed, cut into small pieces and dried in the shade at room temperature, away from sun light. During the drying process, they were turned over to allow homogeneous drying. After this period, they were grinded by a grinder to obtain a powder form and then preserved in a container away from light, heat, and moisture for later use.

2.2 Preparations of Crude Extracts

Powdered leaves and stems of *A. nigellifolius* (10 g) were put into a flask with 150 ml of ethanol and the mixture was then extracted by ultrasound for 30 minutes. After that, the extract was filtered by Buchner funnel under reduced pressure to remove insoluble residues. The filtrates were then condensed with a rotary evaporator to half evaporation and then dried in nitrogen generator.

2.3 Phytochemical Screening

To study the content of the ethanolic extract of *A. nigellifolius* in primary and secondary metabolites, a qualitative detection (Table 1) was performed according to Nasser et al. [13].

2.4 Quantification of Secondary Metabolites

2.4.1 Total phenolic content (TPC)

The Folin–Ciocalteau reagent method has been used to estimate the total phenolic quantities according to Farhan et al. [14]. Five concentrations of the ethanolic extract of *A. nigellifolius* have been prepared and then 100 µl from each concentration have been taken and mixed with 0.5 ml of Folin–Ciocalteau reagent (1/10 dilution) and 1.5 ml of Na_2CO_3 (2%). The blend was incubated in the dark at room temperature for 15 minutes. The absorbance of blue-colored solution of all samples was measured at 765 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg of Gallic acid equivalent (GAE) per g of dry weight of plant powders.

2.4.2 Total flavonoids content (TFC)

The aluminum chloride method was used according to Quettier-deleu et al. [15] for the determination of total flavonoids content of the ethanolic extract of *A. nigellifolius*. 1 ml of various concentrations was mixed with 1 ml of 2% methanolic aluminum chloride solution. After an incubation period at room temperature in the dark for 15 minutes, the absorbance of all samples was determined at 430 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg per g of rutin equivalent (RE) and selected solvent was used as blank.

2.4.3 Total alkaloids

The quantification method for alkaloids determination has been used according to Harborne [16]. 100 ml of acetic acid (10%) in ethanol was added to 1 g of dry powdered plant and then the extract was covered and allowed to stand for 4 hours. After that, the extract has been filtrated and concentrated on a water bath to 25 ml of its original volume. The droplets of concentrated ammonium hydroxide were added to the extract until the precipitation of the whole solution was allowed to settle, and then the precipitates were washed with dilute ammonium hydroxide and then filtered using filter paper Whatman. The residue was dried in the oven at 40℃ and weighed. The alkaloid content was determined using the following formula:

% alkaloid = [final weight of the sample / initial weight of the extract] × 100

2.4.4 Total ash

Powdered leaves and stems (1 g) were put in and burned in a furnace burning (muffle furnace) at 550℃ for 5 hours till the obtaining of an ovary gray color of the powders. Then, the residues have been weighted and the percentage of ash has been estimated according the essential dry weight of plant powder [17].

Total ash content = (final weight / initial weight) ×100

2.4.5 Total lipids

Powders from leaves and stems of plant (10 g) were added in ultrasound apparatus with 200 ml of petroleum ether (40-60℃) and extracted during 1 hour. After that, the solvent was filtered using a Buchner funnel under reduced pressure, and then an evaporation using a rotary evaporator at 40℃ has been done. Finally, the weight of lipids has been calculated.

Table 1. Detection of primary and secondary metabolites

Metabolites	Added reagent	Expected result
Alkaloïds	Dragendorff reagent	Red-orange Precipitate
Tannins	$FeCl_3$ (1%)	Blue color
Resines	Acetone + water	Turbidity
Saponines	Agitation	Formation of Foam
Phenols	$FeCl_3$ (1%) + $K_3(Fe(CN)_6)$ (1%)	Blue-green
Terpenoïds	Chloroform + H_2SO_4 conc	Reddish brown
Flavonoïds	KOH (50%)	Yellow color
Carbohydrates	α-naphtol + H_2SO_4	Purple ring
Reducing sugar	Fehlings (A+B)	Brick red precipitate
Quinones	HCl conc	Yellow precipitate
Sterols et Steroïds	Chloroform + H_2SO_4 conc	Red (surface) + greenish yellow fluorescence
Cardiac glycosides	Glacial acetic acid + $FeCl_3$ (5%) + H_2SO_4 conc	Rings
Diterpenes	Copper acetate (or sulfate)	Emerald green
Anthraquinones	HCl (10%) + chloroform + Ammonia (10%)	Pink color
Proteins & aminoacids	Ninhydrin 0.25%	Blue color
Lignines	Safranine	Pink color
Phlabotannins	HCl (1%)	Blue color
Anthocyanines	NaOH (10%)	Blue color
Flavanones	H_2SO_4 conc	Purple red color
Fixed oils and fats	Spot Test	Oil stain

2.5 Biological Activities

2.5.1 DPPH radical scavenging activity

The method of Rammal et al. [18] has been used for the scavenging ability of DPPH antioxidant test. Increasing concentrations of the ethanolic extract were prepared. 1 ml of different concentrations of diluted extract was added to 1 ml of DPPH. The reaction mixtures were mixed very well by hand and then incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The DPPH scavenging ability of plant extracts was calculated using the following equation:

$$\% \text{ scavenging activity} = [(\text{Abs control} - \text{Abs sample})]/ (\text{Abs control})] \times 100$$

Control= 1 ml DPPH+ 1 ml ethanol
Blank= 1 ml ethanol

2.5.2 Antiproliferative activity

The A549 human lungs adenocarcinoma were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin / streptomycin (PS) in an atmosphere of 5% CO_2 at 37℃.

Powdered leaves and stems of *A. nigellifolius* were dissolved in dimethyl sulfoxide (DMSO) solvent, as a concentrated stock solution (181 mg/ml), and then filtered to be used for the preparation of different concentrations for the treatment of cells.

A total of 50 to 60% confluent cells were treated with *A. nigellifolius* extract for 24 hours in complete medium, seeded in a 24-well plate in a concentration of 10^5 cells/ well. The evaluation of the antiproliferative activity was performed by measuring cell viability of the cell line A549 after treatment for 24 hours with increasing concentrations of both *A. nigellifolius* extract (1, 2, 4, and 8 mg/ml) and CDDP (4, 8, 12 and 80 µg/ml). Similarly, the A549 cells were treated with combinations of the *A. nigellifolius* extract and CDDP.

2.6 Neutral Red Uptake Assay

The absorption test of the neutral red provides a quantitative estimate of the number of viable cells in a culture. It is one of the most used with many biomedical and environmental applications cytotoxicity tests. It is based on the ability of viable cells to incorporate and bind neutral red dye in supravitals lysosomes.

Medium was aspirated and cells were washed twice with PBS (1x), and incubated for 3 hours in a medium supplemented with neutral red (4 mg/ml).

On the other hand, the acetic acid used for cell lysis acid solution, Medium was washed off rapidly with twice with PBS (1x), cells were subjected to further incubation of 20 minutes at 37℃ in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. And then the cells were transferred to a 96 well plate (200 µl/well) in order to read the optical density using a multiwell microplate reader (Thermo Scientific, USA) at 490 nm wavelengths.

2.7 Statistical Analysis

Data were analyzed by using PRISM software. Statistical significance was determined by one way-ANOVA followed by Benferoni test and represented as a mean ± S.E.M. Differences in *p*-values were considered significant if $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

Phytochemicals are bioactive molecules of plants that are involved in the protection of human health against chronic degenerative diseases. Our results (Table 2) showed that *A. nigellifolius* ethanolic extract contained active compounds such as polyphenols, saponin, alkaloids, tannins, flavonoids, terpenoids and glycosides.

The phytochemical screening plays a good role in the estimation of a possible involvement of this plant in the prevention or treatment of some diseases. Saponin and terpenoids were used as anticancer, antioxidant, antimicrobial, anti-yeast, anti-inflammatory and antiviral compounds. Alkaloid and tannins were reported to exert important anticancer and antioxidant activities [19].

In the same manner, flavonoids were reported to produce several biological effects and their apoptosis inducing activities were identified in a previous study [19].

3.2 Active Contents

The percentage of different compounds found in *A. nigellifolius* is presented in the Table 3. The obtained results indicated that this plant contains alkaloids in moderate amount. This latter is known to exhibit analgesic and antibacterial activities [20]. Also, the studied plant contains a percentage of lipids.

3.3 TPC and TFC

The TPC and TFC of the ethanolic extract of *A. nigellifolius* were estimated using Folin Ciocalteau and aluminum chloride assays respectively. Ethanolic extract contained 86.7 mg/ml TPC and 50.2 µg/ml of TFC.

There is a close correlation between the amounts of TPC and TFC and the scavenger activity of extracts. When the quantities of phenol are high, the antioxidant activity of extract is high [21].

3.4 Antioxidant Activity

Polyphenols are very significant compounds generated by plant. Epidemiological studies have shown the role of these compounds in the defense against neurodegenerative diseases, cardiovascular and tumor, in addition to their antioxidant activity [22]. Inhibition or prevention of the oxidation of oxidizable materials in scavenging free radicals and thus limit the damage caused by the oxidation of nitrogen from oxygen or stress.

DPPH method was used to monitor the antioxidant activity of plant extract. The obtained results (Fig. 1) showed that the ethanolic extract from this plant had high antioxidant activity which reached 93.5% at the dose 0.5 mg/ml with an IC_{50}=0.178 mg/ml. This result may be due to the presence of high amounts of total phenolic and total flavonoids.

3.5 Cell Viability Results

The effect of *A. nigellifolius* ethanolic extract on the A549 cell line was tested. The obtained results indicated that treatment with the ethanolic extract caused a decrease in the viability of A549 cells in a dose-dependent manner.

Table 2. Phytochemical screening tests of the ethanolic extract of *A. nigellifolius*

Alkaloids	Tannins	Saponin	Phenols	Terpenoids	Flavonoids	Glycosides
+++	++	+	+++	+++	+++	+

+++ = high amount; ++ = moderate amount; and + = low amount

Cell viability begun to decrease at the concentration 1 mg/ml and the concentrations of 4 and 8 mg/ml has significantly decreased the viability of the cells compared to untreated cells (control) (Fig. 2).

Table 3. Active contents in *A. nigellifolius*

Total alkaloid	Total ash	Total lipids
1.13%	13.88%	30%

3.5.1 Treatment with CDDP during 24 hours causes a decrease of the cell viability of A549 in a dose- dependent manner

The cell viability was measured after 24 hours of treatment with the CDDP and applying different concentrations (4, 8, 12 and 80 µg/ml). CDDP caused a significant decrease in cell viability compared to untreated cells (Control) at a concentration 12 µg/ml (46% mortality of cells). No significant difference between control by comparison with the other two concentrations (4 and 8 µg/ml) and there is no significant difference between a concentration of 12 µg/ml and the concentration of 80 µg/ml (Fig. 3).

3.5.2 Effect of combination of *A. nigellifolius* extract with CDDP on A549

Concentration of 2 mg/ml of ethanolic extract was chosen for all subsequent experiments. Neutral red results showed no significant decrease in cell viability when cells were treated with a combination of 2 mg/ml of ethanolic extract and CDDP 4 or 8 µg/ml compared to the control or to the CDDP alone (Fig. 4).

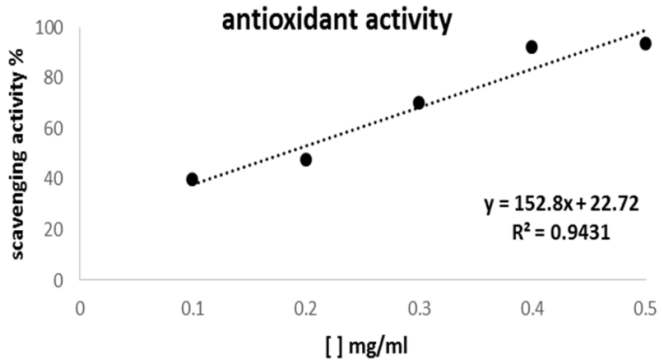

Fig. 1. Scavenging activity (%) versus concentration of ethanolic extract (mg/ml) of *A. nigellifolius*

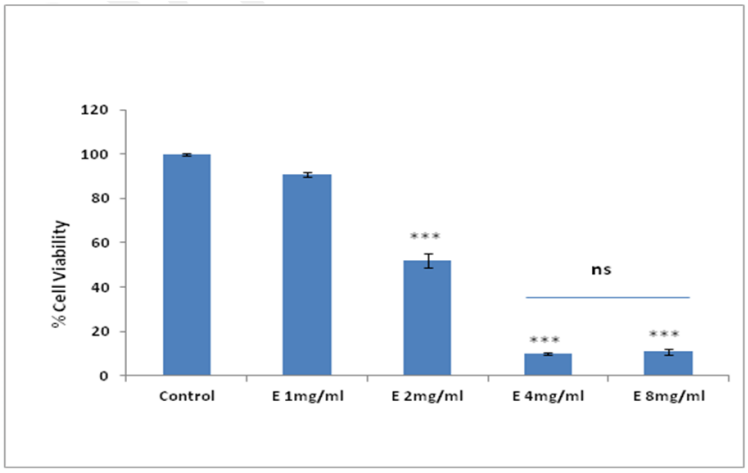

Fig. 2. Effect of ethanolic extract of *A. nigellifolius* on the cell viability of A549
*n=6; (***:P <0.001), ns: no significant difference; treatment during 24 hours (n:replicats)*

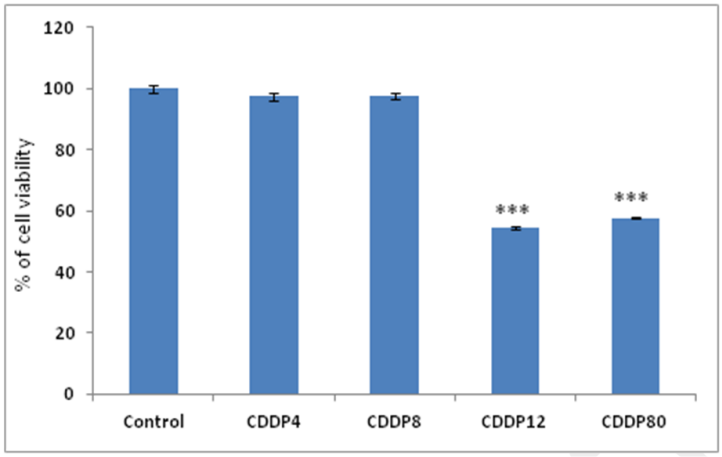

Fig. 3. Effect of CDDP on the cell viability of A549
*n=11, ***:P<0.001*

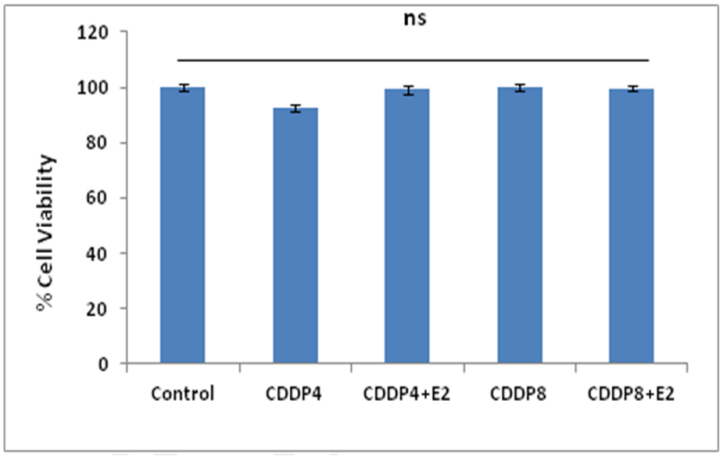

Fig. 4. Effect of the combination of *A. nigellifolius* (2 mg/ml) and CDDP (4 and 8 µg/ml) on the cell viability of A549
n=11; ns = no significant difference between the Control and another treatment

3.5.3 Effect of combination of ethanolic extract of *A. nigellifolius* (2 mg/ml) with CDDP (12 µg/ml) on the cell viability of A549

Ethanolic extract (2 mg/ml) and CDDP (12 µg/ml) showed a significant decrease of cell viability compared to the control (44% and 53% respectively). When the ethanolic extract (2 mg/ml) was combined to CDDP (12 µg/ml) the cell viability was increased and no significant difference was appeared between the untreated cells (Control) and the combination (Fig. 5).

In this study, we evaluated the response of human cells NSCLC (A549) against the alone *A. nigellifolius* ethanolic extract alone and combined with a low dose of chemotherapeutic drug: CDDP.

We have showed that *A. nigellifolius* extract decreased the cell viability of the A549 cells in a dose dependent manner after 24 hours of treatment compared to the untreated cell control.

Patients treated with CDDP, alone or in combination with other chemotherapeutic drugs, suffer from side effects. Hence the idea is to minimize the dose of CDDP and compensate it by the combination with the *A. nigellifolius* extract, and then minimize side effects. So, when combined *A. nigellifolius* ethanolic extract with a low dose of CDDP, decreased viability of A549 cells was not significant in comparison with the cell viability of the groups treated with chemotherapy alone or the only *A. nigellifolius* extract. Then this latter does not increase the anticancer effect of low-dose CDDP.

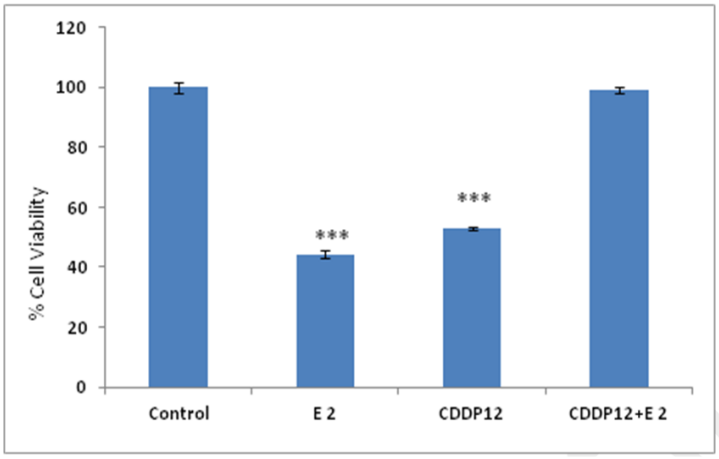

Fig. 5. Effect of the combination of ehanolic extract (2 mg/ml) and CDDP (12 µg/ml) on the cell viability of A549

****: p<0.001; n=5*

4. CONCLUSION

The objectives of our present work were to determine the content of the ethanolic extract from leaves and stems of fresh Lebanese *Anacyclus nigellifolius* Boiss in primary and secondary metabolites and to evaluate its antioxidant and anti-tumor activities on Human lung A549 adenocarcinoma cells.

Our obtained results demonstrated that *A. nigellifolius* has an antiproliferative effect on the viability of A549 cells. In addition, the combination of the *A. nigellifolius* with a low dose chemotherapeutic exhibits a lacking synergistic inhibitory effect against cell viability of non-small cell lung cancer.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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