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Review Article

In Vitro Evaluation of the Activity of *Punica granatum L.* Aqueous and Methanol Extracts on the Trophozoites of *Entamoeba histolytica*

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Abstract

Background: *Entamoeba histolytica* is the causative agent of amoebic dysentery and hepatic abscesses. Despite the efficacy of metronidazole in alleviating infectious diseases, the global dissemination of drug-resistant parasites raises the possibility that *Punica granatum* could serve as an effective natural alternative treatment. ***Objective***: To evaluate the effect of *P. granatum* methanolic and aqueous extracts of various parts against *E. histolytica* trophozoites in an *in vitro* setting. ***Methods***: Various concentrations (0.14, 0.7, 1.4, and 2.8 mg/ml) of *P. granatum* extracts of the flowers, leafs, peels, and seeds were chosen for this purpose. A culture medium containing $0.05x10^6$/ml *E. histolytica* trophozoites was treated with different concentrations of these extracts. The incubation period was 48 hours at 37°C. For every set, an untreated control was also performed. The standard medication metronidazole (17 µg/ml) was employed as a comparative control. ***Results***: All parts of *P. granatum* showed high efficacy against *E. histolytica* trophozoites, but utilization of the methanolic extract proved to be quite effective compared to aqueous extract. Under a light microscope, several morphological changes were also seen. These include changes to the plasma membrane, reorganization of vacuoles that hold cell waste, and major changes to the cytoplasmic granules. ***Conclusions***: The leaf, seed, flower, and peel extracts of *P. granatum* effectively inhibit the growth of *E. histolytica* trophozoites *in vitro*. The use of methanolic extract was more effective compared to the aqueous extract, and can be used as a natural alternative treatment for amoebic dysentery.

Keywords: Amebiasis, Dysentery, *Entamoeba histolytica*, Plant extracts, *Punica granatum.*

تقييم فعالية المستخلص المائي و الميثانولي لنبات الرمان على الاطوار المتغذية للاميبا *Entamoeba histolytica* في المختبر

الخلاصة

الخلفية: يعد طفيلي الاميبا الحالة للنسج *E. histolytica* المسؤول عن تطور الزحار الأميبي وخراجات الكبد. و على الرغم من أن عقار الميترونيدازول ساعد في التخفيف من آثار الأمراض المعدية، فقد ظهرت طفيليات مقاومة للأدوية عالميًا، لذلك سلطت الاضواء على النباتات الطبية كأدوية طبيعية بديلة، مما ولّد الاهتمام بالرمان كا أحد النباتات المهمة.. **الهدف**: تقييم تأثير مستخلص الرمان ضد الأطوار المغذية لطفيلي الأميبا في بيئة مختبرية. **الطريقة**: بعد عدة تجارب، تم اختيار تراكيز مختلفة (0.14، 0.7، 1.4، و 2.8 مجم/مل) من مستخلصات الرمان للزهور والأوراق والقشور والبذور. تمت معالجة وسط استزراع يحتوي على 0.05×10^6مل من تروفوزويتات *E. histolytica* بتراكيز مختلفة من هذه المستخلصات. كانت فترة الحضانة 48 ساعة عند 37 درجة مئوية. لكل مجموعة ، تم إجراء تحكم غير معالج أيضا. تم استخدام الدواء القياسي ميترونيدازول (17 ميكروغرام/مل) كعنصر تحكم مقارن. **النتائج**: أظهرت النتائج بان جميع أجزاء نبات الرمان ذات فعالية عالية ضد الاطوار المتغذية للاميبا الحالة للنسج ولكن استخدام المستخلص الميثانولي أثبت فعاليته مقارنة بالمستخلص المائي. علاوة على ذلك، تم اكتشاف العديد من الاختلافات المورفولوجية تحت المجهر الضوئي، والتي تتضمن حدوث تغيرات في غشاء البلازما، وإعادة تنظيم الفجوات التي تحتوي على المخلفات الخلوية، وحدوث تعديلات كبيرة في الحبيبات السيتوبلازمية. **الأستنتاج**: أثبتت الدراسة إن مستخلصات الأجزاء المختلفة من نبات الرمان ذات فعالية في إيقاف نمو الاطوار المتغذية الحالة للنسج للاميبا في الوسط الزرعي، ومن الممكن استخدامه كعلاج للزحار الاميبي.

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INTRODUCTION

Amebiasis is caused by the intestinal protozoan *E. histolytica*, while transmission occurs via ingestion of food and water contaminated with mature amoebic cysts [1,2]. *E. histolytica* is regarded as the third-greatest parasitic cause of human mortality, following malaria and schistosomiasis, particularly in underdeveloped nations with poor sanitation [3]. The World Health Organization (WHO) estimates that there are more than 50 million cases of amebiasis each year, along with between 40,000 and 110,000 fatalities. However, approximately only 10% of these instances are symptomatic, while the remaining cases are asymptomatic, and the infection varies from nation to nation [4]. Metronidazole, a synthetic nitroimidazole, exhibits efficacy against amebiasis. The emergence of metronidazole resistance in protozoa and helminths is a significant and growing public health issue. Additionally, numerous undesirable effects have been reported during metronidazole treatment, including headaches, dizziness, vomiting, and a metallic taste in the mouth [5]; furthermore, it has been associated with the development of pancreatitis, neurotoxicity, and neutropenia [6]. Experimentally, metronidazole has been discovered to induce genetic mutations in bacteria and lead to the development of cancer in mice and rats when given in high dosages over long periods [7]. Therefore, Philip *et al.* [8] noted that the identification of new therapies to combat the resistance of *E. histolytica* to metronidazole is imperative. Thus, global interest in medicinal plant research as a source of pharmacologically active chemicals has expanded [9]. One such species is *Punica granatum*, or pomegranate, which belongs to the Punicaceae family. It is an ancient fruit known to man, mentioned in the Quran and other religious texts [10]. The pomegranate harbors a diverse array of vital therapeutic and nutritional components; alkaloids are present in several locations, such as the root, tree bark, and fruit peel. Pomegranate seeds and juice contain significant amounts of antioxidants, such as polyphenols. Moreover, the fruit is abundant in vitamin C, vitamin K, and potassium, while the pomegranate seed is mainly composed of hydroxybenzoic acid. In addition, the other compounds present in pomegranate include sterols, tocopherols, triterpenes, isoflavones, phenyl aliphatic glycosides, conjugated fatty acids, non-conjugated fatty acids, and lignins [11,12]. *P. granatum* has several medicinal uses, including the treatment of kidney stones, painful urination, diarrhea, dysentery, anemia, hemorrhoids, etc. [13,14]. The extract of *P. granatum* frequently inhibits biofilm formation by foodborne pathogenic bacteria [15]. Other studies have indicated that the leaf, peel, and exocarp of the pomegranate have antibacterial characteristics that can combat *Escherichia coli*. Pomegranate peels have been identified as possessing antibacterial and antifungal effects. The antibacterial activity of peel extract was investigated using three bacterial strains, namely *Staphylococcus aureus, E. coli, and Salmonella enteritidis*. The antifungal efficacy showed limited effectiveness against two strains of fungi belonging to the *Aspergillus* species [16]. Calzada *et al*. conducted *in vitro* testing on 26 plant species often used in traditional medicine to treat digestive diseases in order to examine the potential anti-protozoan activity of methanolic extracts produced from various plant species. *P. granatum* was one of the plants used, and it showed significant efficiency against *E. histolytica* [17]. In additional investigations, the alcoholic extract of *P. granatum* pulp was found to have anti-amoebic action against *E. histolytica*, whilst the oils and juice of *P. granatum* demonstrated anti-amoebic activity and a synergistic effect with metronidazole [18]. It also exhibited an anthelmintic effect against veterinary endoparasites *in vitro*, and its extract completely eradicated experimental parasites [19]. Other research showed that pomegranate peel extract was effective in preventing and treating *Giardia lamblia* infection [20]. In Iraq, the growing instability, the severe scarcity of medicines, the contamination of food due to preparation and marketing in an unhealthy manner, and the breeding of domestic animals have all contributed to the increased prevalence of *E. histolytica*. Hence, it was imperative to discover treatment alternatives to combat the parasite *E. histolytica.* The current study aimed to assess the efficiency of methanolic and aqueous extracts of *P. granatum* peel, leaves, flowers, and seeds against *E. histolytica* trophozoites *in vitro*.

METHODS

Sample collection

The samples were taken from patients who had bloody diarrhea and abdominal pain and were suspected of being infected with *E. histolytica*. Plastic containers were used to collect stool samples, and direct wet mount preparation and iodine staining were used to confirm infection with them.

Cultivation of parasite

The *E. histolytica* trophozoites were cultivated xenically and maintained in diphasic modified Locke's egg (LE) medium for 48 hours at 37°C. The antibiotics procaine benzyl penicillin (100 IU/ml), streptomycin sulfate (2 mg/ml), and nystatin (2 mg/ml) were added to the LE medium [21]. At 48 and 72 hours after inoculation, trophozoites were harvested in log-phase growth and counted using a hemocytometer.

Preparation of Extracts

Punica granatum samples were obtained from the local market in Baghdad and then authenticated in the Department of Biology, College of Science for Women, University of Baghdad. To prepare *P. granatum* extract, the plant was washed with tap water, shade-dried, and crushed to prepare it for extraction with a Soxhlet apparatus at a ratio of 1:10 (weight:volume) for a duration of 6–8 hours at a temperature of 60–80 ºC. The solution was filtered

using Whatman paper number 1 and dried, and the residue was weighed to prepare a stock solution.

Phytochemical tests

For the qualitative analysis of phytochemicals, chemicals derived from *P. granatum* were extracted, screened, and identified. The analysis was undertaken in the Department of Biology, College of Science for Women, University of Baghdad, to detect substances such as alkaloids (Mayer's and Winger's reagents), phenols ($FeCl_2$ and $PbHCO_3$), trepenoids (chloroform and H_2SO4), tannins ($PbHCO_3$), flavonoids (Mg-HCl and H_2SO_4), and saponins ($HgCl_2$, foam).

Anti-parasitic activity and mortality rate

Different concentrations of *P. granatum* extracts were selected after a series of trials. The doses were determined to be 0.14, 0.7, 1.4, and 2.8 mg/ml for each plant part (flower, leaf, peel, and seed, respectively). *E. histolytica* trophozoites (0.05 x 10^6/ml) was inoculated into a culture medium treated with *P. granatum* extract in different concentrations over three iterations and then incubated at 37°C for 48 hours. A control (untreated) was also prepared for each set of experiments, and the standard drug metronidazole (17 µg/ml) was also used in the study [21]. The effectiveness of *P. granatum* extract was determined using a light microscope to evaluate the growth and morphology of trophozoites and to determine the mortality rate using a Neubauer counter-cell chamber. The following formula was used to calculate the mortality rate [22]:

Mortality rate (%) = 100 – treated / untreated control x 100

Statistical analysis

The data were statistically examined using SPSS version 20. Results were presented as the mean plus or minus the standard error. A one-way ANOVA test was used to find differences between means. *Post hoc* analysis (Least Significant Difference and Duncan test) was used to see if the results were statistically significant. Differences were considered statistically significant at a *p*-value less than 0.05.

RESULTS

Chemical tests on the plant showed that both the water- and methanol-based extracts of *P. granatum* contained alkaloids, phenols, trepenoids, flavonoids, tannins, and saponins. All phytochemicals were found in all parts of *P. granatum* (flower, leaf, fruit peel, and seeds), as shown in Table 1.

Table 1: Phytochemicals detected in the extracts of crude *P. granatum*

Detected Phytochemicals	Plant parts (ME and AE)			
	Flower	Leaf	Peels	Seed
Alkaloids	+	+	+	+
Phenols	+	+	+	+
Trepenoids	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Saponins	+	+	+	+

ME: methanolic extract; AE: aqueous extract.

In this work, we investigate the anti-amebic activity of crude aqueous and methanolic extracts derived from *P. granatum*. Table 2 summarizes the anti-amoebic activity of *P. granatum* extracts on *E. histolytica* trophozoites. All parts of the plant extracts (leaf, seed, flower, and peel) showed significant anti-amoebic activity on *E. histolytica* with a high mortality rate, especially in the methanolic extract.

Table 2: Activity of *P. granatum L.* extracts on *E. histolytica* trophozoites

Treatments	Concentrations of extract (mg/ml)				Control
	0.14	0.7	1.4	2.8	
	Trophozoites x 10^6				
Leaf (AE)	1.765±0.235^{a}	1.290±0.16^{a,b}	0.905±0.135^{b}	0.140±0.000^{d}	1.895±0.075^{a}
Leaf (ME)	1.425±0.025^{a}	0.805±0.045^{b}	0.640±0.11^{b}	0.140±0.03^{c}	1.895±0.075^{d}
Seed (AE)	1.480±0.32^{a}	0.500±0.3^{b}	0.105±0.025^{b}	0.050±0.00^{b}	1.895±0.075^{a}
Seed (ME)	1.355±0.015^{a}	0.935±0.115^{b}	0.680±0.08^{b}	0.100±0.00^{c}	1.895±0.075^{d}
Flower (AE)	0.975±0.105^{a}	0.605±0.015^{b}	0.430±0.03^{b,c}	0.320±0.05^{c}	1.895±0.075^{d}
Flower (ME)	0.480±0.02^{a}	0.440±0.16^{a}	0.200±0.00^{a,b}	0.105±0.005^{b}	1.895±0.075^{c}
Peel (AE)	0.725±0.325^{a}	0.320±0.08^{a,b}	0.140±0.01^{b}	0.100±0.00^{b}	1.895±0.075^{c}
Peel (ME)	0.290±0.02^{a}	0.110±0.01^{b}	0.080±0.01^{b}	0.045±0.005^{b}	1.895±0.075^{c}

Values were expressed as mean±standard error. AE: Aqueous Extracts; ME: Methanol Extracts; values with different superscripts (a,b,c,d) indicate a significant difference (p<0.05) between the mean concentrations of each part of *P. granatum*.

The methanol peel extract showed a high inhibition potency (0.045±0.005 x 10^6 trophozoites) at a concentration of 2.8 mg/ml compared with all treatments and was close to the control drug metronidazole (0.01±0.000 x 10^6 trophozoites). In aqueous extracts, *P. granatum* seeds were more active than methanolic extracts (0.050 ± 0.00 x 10^6 *vs*. 0.140 ± 0.03 x 10^6 trophozoites), with a high death rate. In addition, methanolic extracts of leaf, seed, flower, and peel show high activity and mortality rates against *E. histolytica*.

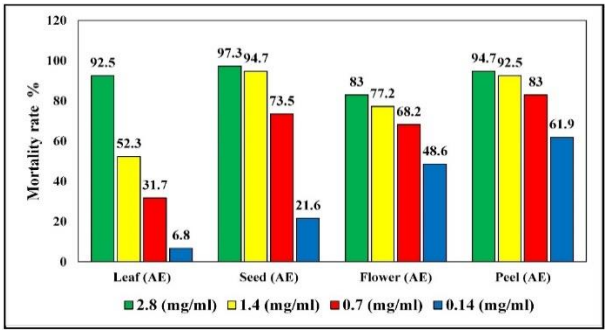

Figure 1: Percentage mortality of *E. histolytica* trophozoites after treatment with aqueous extracts (AE) from *P. granatum* (peel, leaves, flowers, and seeds).

The concentration of 0.14 mg/ml showed low and moderate activity on both extracts, with mortality ranging from 6.8% to 61.9% for aqueous extracts and 24.8% to 84.6% for methanol extracts (Figures 1 and 2).

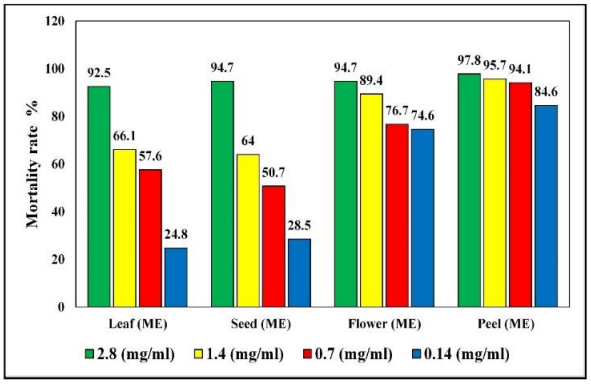

Figure 2: Percentage mortality of *E. histolytica* trophozoites after treatment with methanol extracts (ME) from *P. granatum* (peel, leaves, flowers, and seeds).

Under the microscope, the effects of several *P. granatum* extracts on the formation of *E. histolytica* trophozoites were investigated. Based on the analysis, it was found that *E. histolytica* trophozoites were affected in different ways depending on the type of extract (aqueous and methanol extracts) and the part of the plant (flower, leaf, peel, or seed). The results demonstrated that trophozoites treated with flower methanol extract showed changes in the plasma membrane and in the redistribution of vacuoles with cellular debris, while trophozoites treated with an aqueous extract of the flower showed an alteration in trophozoites granularity with a large vacuole. Microscopic analysis also revealed that trophozoites treated with leaf extracts (aqueous and methanol) exhibited morphological changes, including plasma membrane degradation, the development of wrinkles, and surface membrane abnormalities. When trophozoites were treated with methanolic extracts of peel, the parasite's structure changed, and the plasma membrane developed small blebs that extended from the trophozoites. When trophozoites were treated with an aqueous peel extract, a big, spherical vacuole was observed inside the trophozoites. When the samples were treated with seed methanolic extract, significant alterations were observed in the cytoplasmic granules and vacuoles that still contained food remnants. Following treatment with seed aqueous extracts, the cytoplasm was clearly divided into granule and vacuole sections, with a large vacuole visible at the precystic stage.

DISCUSSION

Entamoeba histolytica is the main cause of gastrointestinal infections and can spread through contaminated food or water; indeed, this route of infection is the most prevalent. In both Iraq in particular and worldwide in general, gastrointestinal disorders are among the leading reasons for requests for medical consultations and are therefore considered to be public health concerns.

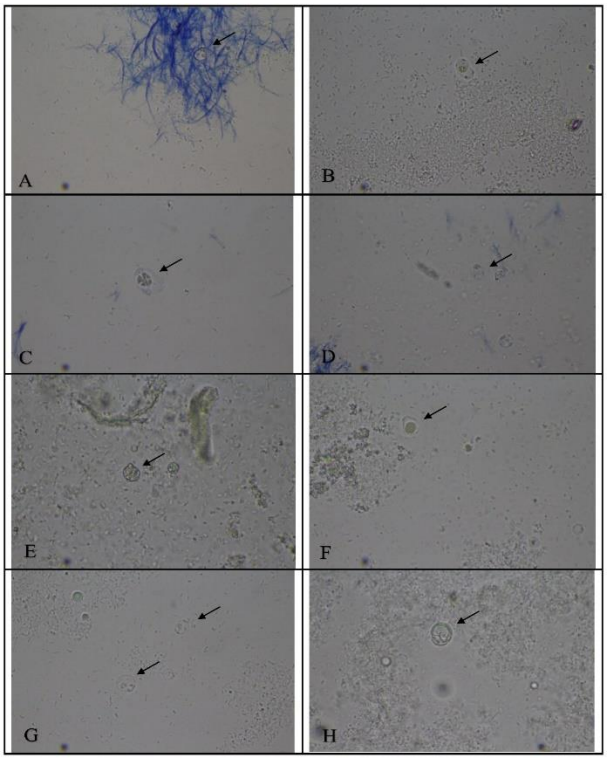

Figure 3: A Light microscope view of the effects of different types of *P. granatum* extract on the growth of *E. histolytica* trophozoites. A&B: flower (ME & AE) treatment C&D: Leaf (ME & AE) treatment, E&F: peel (ME & AE) treatment, G&H: seed (ME & AE) treatment. AE: Aqueous Extracts; ME: Methanol Extracts.

Although these illnesses can impact individuals of any age or gender, infants and the elderly are particularly susceptible [21]. Parasitic illnesses have been a significant global concern for an extended period, particularly in densely populated nations and communities characterized by inadequate infrastructure. Furthermore, over time, there has been an increasing trend of parasites developing resistance to various medications, which exacerbates the issue and diminishes the efficacy of therapies [8]. *Punica granatum* has been used as a medicinal plant since antiquity, as evidenced by its historical use in the treatment of different digestive system and skin diseases. Moreover, it has recently gained the designation of a "superfood" due to its notable abundance of antioxidants, which are believed to assist the human body in combating various diseases [23]. In this study, different parts of *P. granatum* (flower, leaf, peel, and seed) were used after their extraction using aqueous and methanolic solutions, and phytochemical analysis was used to determine the effects of the *P. granatum* plant on the culture of *E. histolytica*. This, in turn, would indicate which parts of the plant had a stronger effect by finding the mortality rate. Our results indicated that *P. granatum* contains a variety of active substances, such as alkaloids, phenols, terpenoids, flavonoids, tannins, and saponins. These

compounds are present in all portions of the plant, and this finding aligns with several studies that have corroborated the presence of various chemical components in different regions of the *P. granatum* plant [24-26]. According to previous studies, these chemicals have a variety of biological activities, including antibacterial, antiviral, antioxidant, anti-inflammatory, and anti-neoplastic effects [14,27,28]. To determine how well *P. granatum* works as a treatment against amoebas, we examined how well *P. granatum* extracts eradicated *E. histolytica* trophozoites. The results showed that methanolic extracts of the pomegranate plant performed significantly better than aqueous extracts. The plasma membrane's structure changed, vacuoles carrying cell waste were reorganized, and cytoplasmic granules underwent significant alterations. There was evidence that metronidazole was very good at stopping the growth of *E. histolytica*, but it was also found that methanol and water extracts had a much bigger effect on the number of cells and their shape. These results were in agreement with various studies that confirmed the therapeutic importance of *P. granatum* as an anti-amoebic. Calzada *et al.* [17] demonstrated the antiprotozoal effect of *P. granatum* against *E. histolytica* in their investigations. Their analyses demonstrated *P. granatum*'s capacity as a safe and efficient natural remedy for diseases caused by *E. histolytica*. Dardona and Al-Hindi [18] conducted a study to examine the impact of alcoholic and aqueous extracts of *P. granatum* peel and pulp, as well as *P. granatum* juice, on *E. histolytica*. Their findings demonstrated that an alcoholic extract of *P. granatum* pulp had the most potent anti-amoebic activity. Although both the aqueous extract and the *P. granatum* juice had anti-amoebic properties, the study found that the alcoholic extract was more effective than the aqueous extract [18]. Furthermore, an abundance of research has demonstrated the efficacy of *P. granatum* in the treatment of various parasitic diseases. It was used as an anthelmintic in the study of Muehlenbachs *et al.* [29]. The authors of this work demonstrated that the use of *P. granatum* peel resulted in a significant decrease in the quantity of viable eggs [29]. These findings also align with recent research that has shown that *P. granatum* extracts have anti-schistosomiasis action against *S. mansoni*. Furthermore, the study by Yones *et al.* [30] suggests that *P. granatum* might be a good choice for developing a new and effective way to treat schistosomiasis because it has effects on the function, mortality, and surface-level morphological abnormalities in adult worms. Also, the effects of *P. granatum* on living protoscolices of *E. granulosus* were demonstrated; the protoscolices died off completely after being exposed to *P. granatum* [31]. The *in vivo* study reported by El-Kady *et al.* also demonstrated that *P. granatum* extract significantly reduced the quantity of *G. lamblia* trophozoites and cysts in the small intestine of infected rats [32]. The data suggest that *P. granatum* could serve as a natural remedy for *G. lamblia* infections. Interestingly, the number of *G. lamblia* in the group treated with *P. granatum* extract was significantly lower than in the metronidazole-positive control group [32]. *Punica granatum* also exhibited significant anti-malarial activity in the host by reducing inflammatory and oxidative stress responses and enhancing the host's inherent immune response. Furthermore, *P. granatum* could serve as an alternative medication to decrease clinical episodes of malaria and potentially prevent infection [33]. As an anti-leishmania agent, *P. granatum* peel extract was tested on *Leishmania infantum*. Imperatori *et al*. showed that the extract suppressed parasite growth and destroyed it via apoptosis. Light transmission and scanning electron microscopy revealed that *P. granatum* extract induced morphological and ultrastructural changes in Leishmania infantum [34].

Conclusion

The leaf, seed, flower, and peel extracts of *P. granatum* were effective in inhibiting the growth of *E. histolytica* trophozoites *in vitro*. Utilization of methanolic extract of *P. granatum* was more effective compared to the aqueous extract. This finding supports the use of this plant for the treatment for amoebic dysentery.

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Conflict of interests

No conflict of interests was declared by the authors.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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