Section A-Research paper

Evaluation The Safety of Black pomegranate (*Punica granatum L.*) Peel Ethanolic Extract

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Abstract

Studies conducted now have validated numerous traditional medicinal plant benefits. Unfortunately, the widespread belief that herbal medicines are extremely safe and free of side effects is not just false. This study sought to determine whether a black pomegranate peel ethanolic extract could have unfavorable effects on rats when given to them in a short amount of time. The extract's effects were assessed in a study of a range findings study. The fruits of black pomegranate (Punica granatum L.) were harvested from orchards In Alkhalis city and Abosyda district -Diyala Governorate, Iraq in September 2021. Dried pomegranate peel was extracted by ethanol 70% containing 1% acetic acid. Phytoconstituent analysis of Peel Extract of Black Pomegranate (BPPE) was carried out by Gas chromatography-mass. The safety of BPPE evaluate by a range-finding study, which is conducted on a total of Twenty-five adult female Wistar rats (Swiss albino rats aged 4-8 months with a weight range 150 to 176 grams. Animals were divided equally into four groups and dosed orally BPPE 2g, 3g, 4g, 5g/kg B.W. of BPPE by stainless gastric gavage needle gauge (18G) for seven consecutive days. When there was, no mortality observed after seven days. The additional five female rats were used and dosed with 15g/Kg, B.W. of BPPE at the same protocol that has been used before. The physical properties of BPPE were dark purple, thick and sticky, had a sour taste and aromatic, the yield extract was (54.9%). The Phytoconstituent exhibited an abundance of esters, furans, Phenols, alkaloids, and saturated fatty acids. The outcome range-finding study revealed that BPEE exhibited no toxic signs but interestingly revealed a significant increase in body weight of all experimental groups that received various dosage rates of BPEE. It could be concluded that is BPEE is practically safe to be administered.

Keywords: Safety ,Black Pumegranate , Punica granatum L,Peel ,Extract

1. Introduction

Since ancient times, plant-derived compounds, so-called phytocompounds, have been traditionally prescribed to prevent or cure some disorders (Aqil et al., 2019), About 80% of people in developing countries rely mainly on traditional herbal medicines for their health care which involves the use of plant extracts or their active substances (Kashyap et al., 2019).

Many traditional effects of medicinal plants have been proven in modern studies (Askari et al., 2020; Eghbali et al., 2021). Currently, this botanical medicine is increasingly becoming popular throughout the world, especially in developing countries, where medicinal plants are available and inexpensive .In addition, the users believe that medicinal plants have less side effects than synthetic drugs. However, the general perception that herbal drug is very safe and devoid of adverse effects is not only untrue, but also misleading. In fact, many studies show that medicinal plants are able to produce a wide range of undesirable and adverse reactions such as carcinogenicity, teratogenicity, life-threatening conditions and even death (Kristanc & Kreft, 2016; Ndhlala et al., 2013).

According to the annual report of American Association of Poison Control Centers (Mowry et al., 2014). Statistical data on people of developing countries suggests an underestimation of that report because the harmful effects of plants is not adequately diagnosed and the information is not accurately reported by the patients (Macías Peacok et al., 2009).

One of the key concerns modern medical practitioners have about medicinal plants is their safety. Researchers have used different laboratory-based assays to measure safety in the use of herbal plants and herbal plants derived products, but challenges remain (Subramanian et al., 2018). For example, although there are many reports on the potential of medicinal plants, there are few studies of the toxicogenic effects when consumed in large amounts (Mensah et al., 2019). In addition, it is not straightforward to compare toxicity data for different plants, given differences in experimental conditions, as well as plant sources. So, Toxicity tests are needed to assess the safety of the drug, or ingredients used as supplements or food (Makiyah & Tresnayanti, 2017).

In study conducted in Iraq by Humadi et al., (2022) they found that Iraqi herbalists in the examined region prescribe and dispense treatments and identify plants differently. Higher-trained herbalists work differently. This difference in practice may pose health concerns to the public; hence, all herbalists should follow a standard. Importing herbal items to distributing them to patients should be regulated (Humadi et al., 2022).

Pomegranate (*Punica granatum* L.), belonging to the Punicaceae family (Holland et al., 2009). Pomegranate is hailed as the ‘fruit of heaven’ in many religious texts and finds mention in the Quran, Bible, Torah and Babylonian Talmud (Viuda- Martos et al., 2010). It is an ancient deciduous tree crop that has been domesticated for thousands of years and offers a broad range of varieties and consumption opportunities for consumers (Chater et al., 2018; Morton, 1987; Preece & Moersfelder, 2016). It is native to the region extending from Iran to the Himalayas in northern India, and Mediterranean and Middle-East countries are currently the main regions of pomegranate cultivation and production and it continues to be popular today (Ferguson, 2002; Jbir et al., 2008; Melgarejo et al., 2009; Rana et al., 2010).

Pomegranate is recognized as the primary source of principal ingredients, including flavonoids, magnesium, potassium, and iron. It also has antioxidant components, alpha-linolenic acid (omega 3), linoleic acid (omega 6), and oleic acid (omega 9) (Alkhatib, 2021).

Pomegranate has been used in traditional medicine for the treatment of diarrhea, dysentery, hemorrhoids, ulcers, intestinal parasites, respiratory complications, sore throat, aphthae,

epistaxis, and vaginal itching and is believed to be tonic for the heart (Askari et al., 2018; Lansky & Newman, 2007; Mohammad & Kashani, 2012). In addition, it has recently been used in the treatment of numerous diseases including diabetes (Faddladdeen & Ojaimi, 2019), Alzheimer's disease (Almuhayawi et al., 2020), cancer (Moga et al., 2021; Turrini et al., 2015), arthritis (Basu et al., 2018), male infertility (ALSHINNAWY et al., 2020), obesity (Michicotl-Meneses et al., 2021), and cardiovascular disorders (D. Wang et al., 2018). Pomegranate seeds are regarded as an excellent natural source of wound healing compounds. (Salim et al., 2022).

There are several types of pomegranate, namely white pomegranate, red pomegranate, and black pomegranate (Setiawati, 2014). Black pomegranates which are characterized by purple to black peel color are known from China in eastern Asia to western Asia (Iran, Afghanistan, Iraq) and differ from each other with respect to fruit characteristics such as taste, growth habit, and dwarfism (Ardekani et al., 2011; Balli et al., 2020; Holland et al., 2009; Özgüven et al., 2010). generally, the color intensity is affected by the chemical structure and concentration of each anthocyanin pigment (Hernandez et al., 1999). Red pomegranate, black pomegranate (Punica granatum L) were reported as important in Iraq.

Black pomegranate has several chemical contents such as saponins, polyphenols, flavonoids, tannins, and alkaloids (Achmad et al., 2019; Rizka & Saptarini, 2018). Since flavonoids such as anthocyanins could be found in red, blue, and purple plant pigments (Mazza & Miniati, 2018). It could be determined that black peel pomegranate has a higher amount of such compounds. Therefore, it could be more efficient for therapeutic purposes (Khorrami et al., 2019). Black pomegranate peel contains the highest total phenolic and flavonoid content than other pomegranate cultivars (Ardekani et al., 2011).

Antioxidants have an essential function in the oxidation of oxidative stress in biological cells caused on by free radicals. (Lazeeza, 2021). The high phenolic content of medicinal plant extracts may be attributed to their antioxidant and antibacterial properties (Hussein et al., 2020). Phenolic compounds are very strong antioxidants, which can neutralize free radicals, and their cytotoxic effects, playing an important role in health (Salimi et al., 2011).

The black pomegranate is a natural medicine for various diseases (Ghazaleh et al., 2013) such as anticancer activity (Taherian et al., 2021), antibacterial (Khorrami, et al; 2019). The antioxidant activity of black pomegranate peel is potent (Chasanah, 2020). Therefore because widely therapeutic potential and not well defined toxicity of black pomegranate (Achmad et al., 2019). We have conducted the current study to define its safety in female rats.

While the widely cited health-beneficial feature of pomegranate have been examined to a certain extent , its unfavorable effects and toxicity , if any, have not been thoroughly studied, possibly due to its standing as a food. The purpose of this research was to investigate into the possible adverse effects of a standardized black pomegranate peel extract after acute administration to rats. In a rang finding study, the effects of the extract were evaluated.

2. Methods

The protocols for the experiment's parts were completed at the University of Baghdad/College of Veterinary Medicine, Department of Physiology, Biochemistry, and Pharmacology. Ethical approval was granted through the local committee of the animal care and use at the college of

Veterinary medicine within the university of Baghdad. (Number at 2022/ / before starting this study.

2.1. Black Pomegranate Fruits Collection

The fruits of black pomegranate (***Punica granatum L.***) were harvested from orchards In Alkhalis city and Abosyda district -Diyala Governorate, Iraq in September 2021 (Figure 1). The plant has been classified by Iraqi national herbarium/Directorate of seed testing and certification (D.S.T.C) / ministry of agriculture, and according to the document No.1182, at date: 8/6/2022.

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Figure1: Black pomegranate tree (Al Khali’s-Iraq).

2.2. Preparation of plant extract

The black pomegranate fruits were washed by running tap water then peels (mesocarp and epicarp) were manually separated, and chopped, they were ambient air-dried at room temperature for 120 –168 h. The dried peels were ground to a powder using an electric grinder (Moonlife, China) before extraction. (Figure 2)

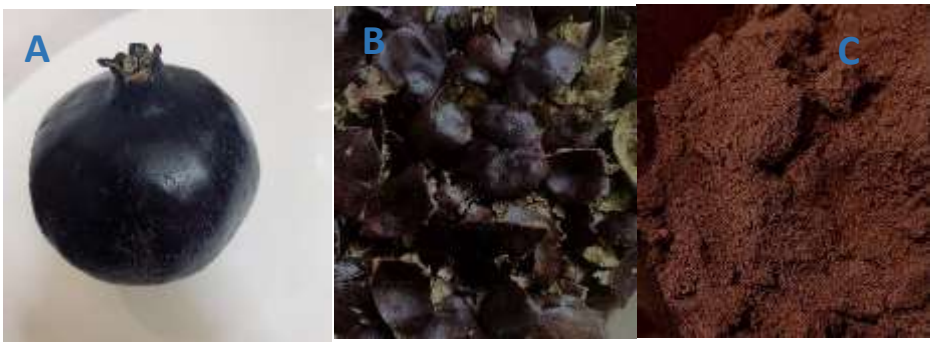


Figure 2: Preparation of plant for extraction. A: Black pomegranate fruit, B: chopped BPP, C: ground BPP.

Dried pomegranate peel was extracted by ethanol 70% (Alpha Chemika, India) containing 1% acetic acid (Alpha Chemika, India) for 72 hours with Soxhlet (Faithful, China) and then filtered through Whatman No. 0.4mm filter paper (CHM, Spain) by a Buckner Funnel (ADARSH , India) under negative pressure.

The extract was vacuum-dried using a rotary evaporator (Heidolph, Germany) (30 –40°C) until the ethanol solvent was removed, and the concentrated extract is stored at freeze temperature until use (Dana et al., 2015).

The yield percentage of BPPE was calculated by using the following equation (Magangana et al., 2021).

$$\text{BPPE\%} = \frac{\textit{weight of BPP extract}}{\textit{weight of crude BPP powder}} \times 100$$

2.3. Phytoconstituent analysis of Peel Extract of Black Pomegranate by Gas chromatography–mass spectrometry GC-MS analysis

Automated pyrolysis gas chromatography combined with a mass spectrometer was used for the GC-MS study of BPPE. (Agelint (7820A) USA)

2.3.1. BPPE preparation for GC-Mass

One mg of the sample has been dissolved in five ml of methanol and then filter by syringe filter 0.2 µm.

3. Experimental design and animals:

Twenty-five adult female Wistar rats (Swiss albino rat) were obtained from Iraqi Center for Genetics and Cancer Research. The rats aged 4-8 months with a weight ranged 150 to 176 grams. Animals were housed in five cages (5 rats per cage) in the animal house of College of Veterinary Medicine. University of Baghdad under appropriate conditions, with a temperature of 20-23 °C. in an air conditioned room to avoid stress factor, and exposed to light for 12 hours daily, with free access to food and water during the experiment, and kept under observation for 2 weeks before the experiment began.

4. Assessment the Acute Toxicity of BPPE by Range Finding Study in Rats

Animals were divided equally into four groups and dosed orally BPPE 2g, 3g, 4g, 5g/kg B.W. of BPPE by stainless gastric gavage needle gauge (18G) for seven consecutive days. When there was no mortality observed after seven days .The additional five female rats were used and dosed 15g/Kg, B.W. of BPPE at the same protocol that has been used before (Lu, 1985).

The BPPE doses were prepared by dissolving 2, 3, 4, 5 ,and 15 grams up to 10 milliliters of distilled water, and each 100 g body weight rat received 1ml of the resultant solutions once a day.

The animal have been monitored for any changes in Body weight, morbidity, mortality, clinical examinations, and signs of toxicity including changes in skin and eyes, breathing issue, seizures, were observed daily, also any abnormal physical and behavioral changes, any changes fur, and autonomic activity like lacrimation, changes in gaits, posture, response to handling, stereotypic activities like excessive grooming, repetitive circling, etc. along the period of this experiment.

5. Results

5.1. Black pomegranate peel extract properties and extraction yield

The physical properties of the product of black pomegranate peel ethanolic extract were dark purple, thick and sticky, had a sour taste and aromatic (Figure 3), which agrees with (Ermawati, 2022). The yield percentage of Black pomegranate peel ethanolic extract was (54.9%).

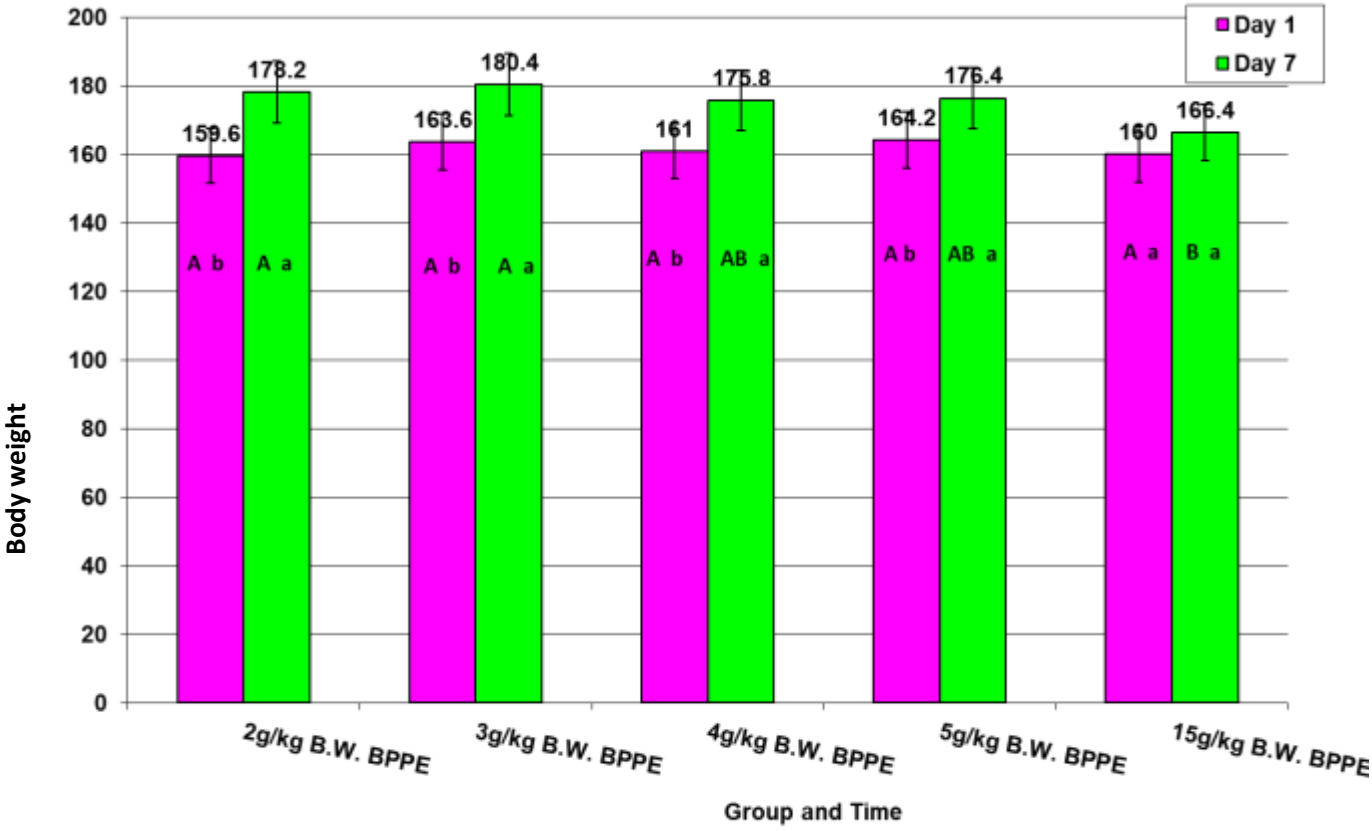
Figure 3: Black pomegranate peel ethanolic extract.

5.2. Phytoconstituent analysis of Peel Extract of Black Pomegranate by Gas chromatography–mass spectrometry GC-MS analysis.

The Phytoconstituent of ethanolic peel extract of black pomegranate exhibited an abundance of esters, furans, Phenols, alkaloids and saturated fatty acids.

5.3. Assessment the safety of BPPE by range findings study.

There were no signs of toxicity as well as clinical or pathological signs, or changes in gastrointestinal behavior that may be attributed to the BPPE administration were observed. No signs of toxicity or deaths were reported, all rats survived and were healthy with a significant $P \leq 0.05$ increase of body weight throughout the trial period (Histogram 1). That is considered to be of no toxicological relevance.



Histogram 1: Effect of dosage rate of black pomegranate peel ethanolic extract on the body weight of female rats. .

Based on these results, the minimum toxic dose of BPPE in rats was established to exceed 15000 mg/kg B.W. Results show that the BPPE is non-toxic even at relatively high dose.

6. Discussion

People may spend a considerable amount of money on different over-the-counter treatments and herbal dietary supplements in order to enhance their overall well-being, and health (Kong et al., 2003; WHO global atlas, 2005; WHO Monographs, 2002).

With the worldwide increase in interest in phytotherapy and phytonutrition, which includes the use of an exponentially expanding number of newly developing herbal supplements, public health concerns about their safety are rising. Despite the promising medicinal and nutritional potential of many herbal species, we must take note that the majority of them have not been thoroughly studied in terms of safety, and the adverse effects associated with their use have not been monitored as rigorously as conventional pharmaceuticals (Traditional Medicine Strategy, 2002).

It has been considered that if a drug is effective, it will have side effects. Therefore, herbal medicines as drugs either have side effects or are ineffective. However, herbal medicines are generally considered safe and effective agents. Therefore, people every year turn to herbal medicine because they believe plant remedies are free from undesirable side effects (George, 2011). There are various kinds of traditional medicines derived from plants and have studied in much chemical content and efficacy in them. But there are still many plants whose toxicity levels are not yet known, so it needs to be further investigated to determine safety ingredient (Abnaz & Levita, 2018).

Pomegranate has been consumed as a medicinal plant in the Middle East for thousands of years (Johanningsmeier & Harris, 2011). Pomegranate possesses various properties such as anti-inflammatory, antioxidant, anti-cancer, anti-diabetic, anti-hyperlipidemic, anti-hypertensive and cardioprotective, antibacterial, and antiviral properties. As well as plays roles in lipid regulation and immunomodulation (Al-Dhaher, 2013; Hassan, 2004; Puneeth & Chandra, 2020; R. Wang et al., 2010). The antioxidant power of compounds found in pomegranate is proven in extensive in vitro and animal studies (Faria et al., 2007). According to Ali & Al-Okaily, (2016) pomegranate juice has been shown to effectively protect the liver from oxidative damage (Ali & Al-Okaily, 2016). Tannins and polyphenols present in pomegranate are considered factors affecting the antioxidant properties of pomegranate. Pomegranate may have anti-lipid peroxidation effects due to the reduction of platelet aggregation, LDL oxidation, and macrophage oxidative status, also decrease serum cholesterol (Hussen, 2014; Pérez-Vicente et al., 2002; Rozenberg et al., 2006).

The black pomegranate has shown natural potential in treating an extensive variety of disorders (Ghazaleh et al., 2013), Due to the presence of tannins, alkaloids, polyphenols, flavonoids and saponins in its chemical composition (Achmad et al., 2019; Rizka & Saptarini, 2018).

Since black pomegranate peel is high in phenolic and flavonoid content (Ardekani et al., 2011), it possesses strong antioxidant properties (Chasanah, 2020). Black pomegranate is also has anticancer (Taherian et al., 2021), antibacterial (Khorrami et al., 2019), and anti-angiogenic (Dana et al., 2012) activity.

Herbal medication safety evaluations have become a significant problem for consumers, regulatory authorities, and health professionals, however investigations of adverse events

associated with these products are much more difficult than analyses of conventional pharmaceuticals (Ekor, 2014).

Nonetheless, it has become clear in recent decades that certain plant-derived substances can cause serious disruptions, such as carcinogenesis, hormonal dysregulation, and disruption of reproductive and developmental processes, even at very low doses (Vandenberg et al., 2012).

Achmad et al. (2019) revealed that black pomegranate peel extract (BPPE) has an acute toxic potential against shrimp larvae (Artemia salina Leach), as shown by LC50 values less than 1000g / ml. According to the Brine Shrimp Lethality Test (BSLT), the LC50 value of BPPE is 114,090 g/ml, indicating the presence of anticancer activity (Achmad et al., 2019).

The edible components of the pomegranate and its extracts are found to be safe to consume. In an acute toxicity trial, a food grade pomegranate fruit extract taken orally had an LD50 of more than 5000 mg/kg in Wistar rats and Swiss albino mice, while in the sub chronic phase, all animals survived with no changes in clinical parameters like behavioral and physical (Patel et al., 2008).

However, due to their high content of alkaloids and tannins, some extracts from other parts may be toxic at high doses. Portion of the pomegranate tree may also impact toxicity; for example, the LD50 of peel extract (intraperitoneally injection of two types from Morocco) was determined to be 320.5-355.8 mg/kg in Wistar rats and 300-348.2 mg/kg in albino mice (Ouachrif et al., 2012). In another study, the acute oral toxicity demonstrated result no evidence of toxicity in a peel extract at a set solitary dosage of 2000 mg/kg for 14 days (El Deeb et al., 2021).

Cerda et al, (2003) conducted a study in which they concluded that giving Wistar rats high doses of punicalagin from pomegranate elagitannins (a commercial diet containing 0.5, 2, 5, 10, and 20% peel extract) for 37 days is not toxic (Cerdá et al., 2003).

In study conducted by Patel et al, (2008) revealed gavage administration of pomegranate fruit extract at levels up to 600 mg/kg/day for 90 days did not affect feed consumption or body weight (Patel et al., 2008). In another study by Cerda et al. (2003), a significant reduction in feed consumption and body weight appeared to be related to high levels of the extract (20%) of punicalagin from pomegranate elagitannins to Wistar rats in the diet for 37 days (Cerdá et al., 2003). Patel et al, (2008) suggested that might be due to different dose and different routes of administration.

A cetain study that is agreed with the finding of the cuurent study cincerning the body weight, found body weight gain increased throughout the test for the young and developing rats. These weight increases were much lower in the groups who received the higher microencapsulated pomegranate juice dosages (2900 and 5000 mg/kg) (Álvarez-Cervantes et al., 2021)

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Other studies unlike the finding of the current study have shown that depending on the amount of polyphenol administered, body weights can be affected. A diet containing 0.4% flavonoid extract from grape fruit (Juskiewicz et al., 2002) or 2% pro-anthocyanidin rich extract from grape seeds (Yamakoshi et al., 2002) did not affect body weight, whereas, administration of diet containing 1% polymeric grape seed tannins (Tebib et al., 1996) or 1.9% catechin (Bravo et al., 1994) resulted in a significant decrease in body weight. It has been suggested that tannins (including

ellagitannins) interact with proteins and inhibit digestion of endogenous protein, which may result in weight loss (Butler & Rogler, 1992). In the present study, administration of black pomegranate peel extract does not significantly affect body weight.

The differential effects on the body weight in these studies is difficult to explain but may be related to differences in the types of polyphenols (Patel et al., 2008).

7. Conclusion

It could be concluded that is the black pomegranate peel ethanolic extract is practically safe to be administered.

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Conflict of Interest: The authors declare there is no conflict of interest.

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