Relevant essential oil components: a minireview on increasing applications and potential toxicity

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10 **Abstract**

11 Phenolic compounds carvacrol, thymol, eugenol and vanillin are four of the most
12 thoroughly investigated essential oil components given their relevant biological
13 properties. These compounds are generally considered safe for consumption and have
14 been used in a wide range of food and non-food applications. Significant biological
15 properties, including antimicrobial, antioxidant, analgesic, anti-inflammatory, anti-
16 mutagenic or anti-carcinogenic activity, have been described for these components. They
17 are versatile molecules with wide-ranging potential applications whose use may
18 substantially increase in forthcoming years. However, some *in vitro* and *in vivo* studies,
19 and several case reports, have indicated that carvacrol, thymol and eugenol may have
20 potential toxicological effects. Oxidative stress has been described as a main mechanism
21 underlying their cytotoxic behavior, and mutagenic and genotoxic effects have been
22 occasionally observed. *In vivo* studies show adverse effects after acute and prolonged
23 carvacrol and thymol exposure in mice, rats and rabbits, and eugenol has caused
24 pulmonary and renal damage in exposed frogs. In humans, exposure to these three
25 compounds may cause different adverse reactions, including skin irritation, inflammation,
26 ulcer formation, dermatitis or slow healing. Toxicological vanillin effects have been less
27 reported, although reduced cell viability after exposure to high concentrations has been
28 described. In this context, the possible risks deriving from increased exposure to these
29 components for human health and the environment should be thoroughly revised.

30 **Keywords:** carvacrol, thymol, eugenol, vanillin, toxicity

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1. Introduction

For centuries, essential oils have been used in traditional medicine, in perfumes and as flavorings and preservative agents in food. In the last few years, they have attracted much attention because of their relevant sensory properties, reported health benefits and consumer demand for natural products (Abbaszadeh et al., 2014). Of the different components responsible for the biological activity of essential oils, phenolic compounds carvacrol, thymol, eugenol and vanillin (Fig. 1) are four of the most popular components as they are widespread in both food and non-food applications, and exhibit a wide range of excellent biological properties (Hyldgaard et al., 2012).

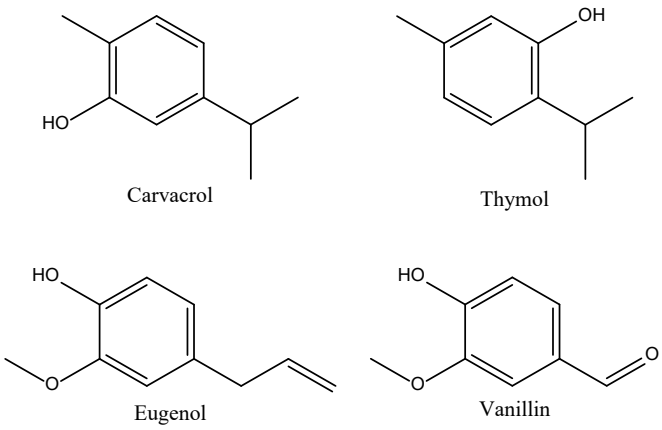

Figure 1. Molecular structure of the EOCs carvacrol, thymol, eugenol and vanillin.

These essential oil components (EOCs) are designed as generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA, 2020), their use as food flavoring is approved in the EU (EC, 2008), and they are considered safe when consumed in commonly used quantities. Indeed, no extensive further toxicological research into these components has been conducted, and a limited number of scientific publications is found in the literature that have evaluated their safety in the last few years.

However, their increasing use in multiple applications as not only pure compounds, but also as a part of plant extracts or spices, together with the high concentrations required to accomplish activity for some applications, may lead to greater consumer exposure to these components (Maisanaba et al., 2015; Nejad et al., 2017).

This short review focuses on four of the most thoroughly investigated EOCs (carvacrol, thymol, eugenol, vanillin), and includes information about their biological properties, current and potential applications, and their toxicological information to clarify any possible risks deriving from prolonged exposure to these components for human health.

2. Methodology

This review was performed in Web of Science, PubMed, Scopus, Espacenet, Google Scholar and Google Patents databases until February 2021. Different combinations of several keywords were applied during the literature research, including: "essential oil component", "carvacrol", "thymol", "eugenol", "vanillin", "pharmacological" "antioxidant", "antimicrobial", "properties", bioactivity", "toxicity", "cytotoxicity", or "safety". No restrictions on language or year of publication were stablished except for the information collected about the prevalence of carvacrol, thymol, eugenol and vanillin research works and patents set in the last 5 years (2016-2020). The European Chemicals Agency (www.echa.europa.eu), the European Commission (www.ec.europa.eu), the U.S. Food and Drug Administration (www.fda.gov), the Grand View Research (www.grandviewresearch.com) and the Mordor Intelligence (www.mordorintelligence.com) websites were also consulted in the search for regulatory and market information.

3. Application outlook of relevant EOCs

The global essential oils market is expected to grow at a compound annual growth rate of 7.5% from 2020 to 2027 (Grand View Research, 2020). The main factor of such growth is increasing consumer demand for natural ingredients in food products due to concerns about adverse health effects related to synthetic preservatives (Mordor Intelligence, 2019). Other key factors include a growing demand for processed foods and beverages, the ever-increasing popularity of exotic flavors and their extended application to industries, such as perfumery, cosmetics, toiletries and aromatherapy (Abbaszadeh et al., 2014; Grand View Research, 2019; Mordor Intelligence, 2019).

Accordingly, the number of research articles about carvacrol, eugenol and thymol has almost doubled in the last 5 years. During this period, more than 2,000 articles about carvacrol and thymol applications have been published and this number rises to 3,000 publications for eugenol. The most active research area of the three above EOCs were agricultural and biological science, biochemistry, pharmacology and medicine. Vanillin research has also drastically increased in recent times, with more than 2,600 research articles published in the last 5 years, whose research has focused on new chemical synthesis methods and biotechnology-based approaches for vanillin production, followed by biochemistry, agricultural and pharmacology applications (Scopus, 2021). The number of patents related to these compounds has also considerably grown in recent years. According to Espacenet, the largest number of patents corresponds to vanillin (14,339), with applications that focus mostly on synthesis and purification methods, although vanillin compositions have also been registered for their use as bactericides, flame retardants or pharmaceutical products to treat different metabolic disorders. Eugenol is the second compound for which more patents have been registered (11,895), followed by thymol (9,057) and carvacrol to a lesser extent (2,954) (Fig. 2). Most of the applications

of these three compounds correspond to antimicrobial compositions, natural food preservatives, feed additives, pesticides, oral care products and pharmaceutical compositions, with a smaller proportion of synthesis methods (Espacenet, 2021). Therefore, they are versatile molecules with wide-ranging biological effects and potential applications that may substantially increase in forthcoming years.

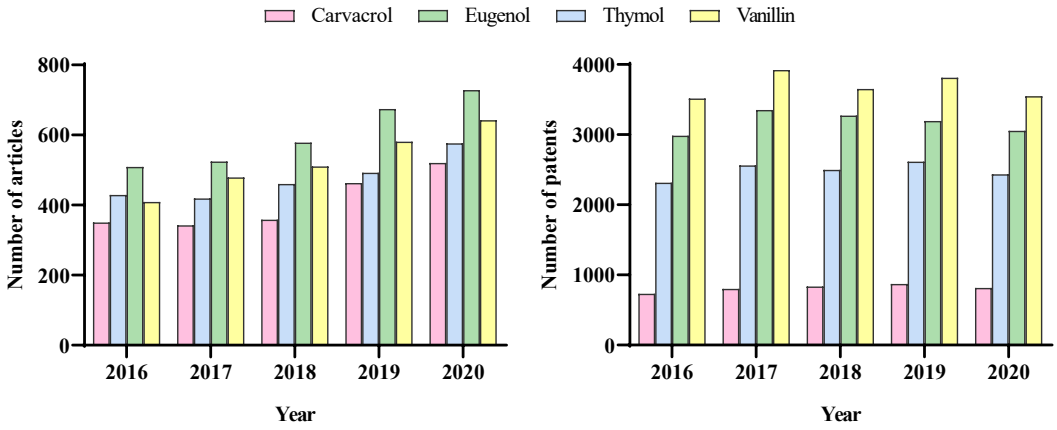

Figure 2. Number of published articles and registered patents for carvacrol, thymol, eugenol and vanillin in the last 5 years (source: Scopus and Espacenet databases).

3. Carvacrol and thymol

Carvacrol (5-isopropyl-2-methylphenol) and thymol (2-isopropyl-5-methylphenol) are two isomeric monoterpene phenols that are found in origanum, thyme, marjoram and other aromatic plants and their essential oil fractions as major components (De Vincenzi et al., 2004). Both these components are used as flavorings in foods, beverages, perfumes, fragrances and cosmetics (Memar et al., 2017). Other applications include their use as disinfectant, insecticide, antiseptic in mouthwash and for dental practice (Kachur & Suntres, 2020; Suntres et al., 2015).

Carvacrol and thymol are two of the most extensively studied EOCs because they have been identified as the most active monoterpenoids against a broad spectrum of microorganisms (Hyldgaard et al., 2012). These components are potent antibacterial agents against Gram-positive and Gram-negative bacteria (Dorman & Deans, 2000; Tippayatum & Chonhenchob, 2007), food spoilage or pathogenic yeast and fungi (Abbaszadeh et al., 2014; Marchese et al., 2016), and have demonstrated the inhibition of toxins production by food-relevant bacteria (Ultee & Smid, 2001). These effects have been proven *in vitro,* and also in different food matrices like meat, fish, dairy products, vegetables, rice, fruit and fruit juice (Burt, 2004; Calo et al., 2015). The antimicrobial activity of carvacrol and thymol has been related to their hydrophobicity and chemical structure, characterized by a hydroxyl group and the presence of a system of delocalized electrons in the phenol aromatic ring (Ben Arfa et al., 2006; Ultee et al., 1999). These elements are responsible for producing significant effects on the structural and functional properties of the cytoplasmic membrane. The main reported antimicrobial mechanism consists in cytoplasmic membrane disruption, which increases its permeability and depolarizes its potential, and leads to intracellular content leakage and bacteria lysis (Xu et al., 2008). Other proposed mechanisms consist in the inhibition of efflux pumps, bacterial motility or membrane bound ATPases, and in the reduction of biofilm formation (Kachur & Suntres, 2020).

These compounds also present a wide range of other beneficial effects. As other phenolic compounds, carvacrol and thymol induce a significant antioxidant effect since their hydroxyl groups act as hydrogen donors, reducing free radical formation and scavenging free radicals (Pereira et al., 2009). Other mechanisms have also been described such the improvement of endogenous antioxidant enzymes, the regulation of intracellular glutathione levels or the synergistic effects with other antioxidant compounds (Aristatile

et al., 2009). Given their antioxidant activity, both components have been proposed as 'natural' replacements for 'synthetic' antioxidant food additives as they minimize oxidation of the lipid components in food (Yanishlieva et al., 1999).

Other reported properties include analgesic, anti-inflammatory, anti-mutagenic and anti-carcinogenic effects, as well as a modulator role in different central neurotransmitter pathways and the immune system (Deb et al., 2011; Salehi et al., 2018; Sharifi-Rad et al., 2018). The protective effects of these components in metabolic disorders like diabetes mellitus, obesity, renal diseases or gastrointestinal disorders, among others, have also been documented (Nagoor Meeran et al., 2017).

Although carvacrol and thymol are generally considered safe for consumption, some studies indicate that they may cause potential toxicological effects and allergic reactions. Table 1 summarizes some of the most relevant *in vitro* and *in vivo* studies performed in the past few years. *In vitro* studies show that both carvacrol and the carvacrol and thymol mixture induce toxic effects on Caco-2 cells when measured by different basal cytotoxicity endpoints. Although no cytotoxic effects have been found for thymol when administered alone, the morphological analysis of exposed cells has shown cellular damage that comes in the form of lipid degeneration, mitochondrial damage, nucleolar segregation and apoptosis (Llana-Ruiz-Cabello et al., 2014). Other authors have reported an IC_{50} value for thymol of approximately 400 μM using V79 and HepG2 cells, while Caco-2 cells prove more resistant to thymol exposure with an IC_{50} value of 700 μM (Slamenová et al., 2007). Oxidative stress seems to play a crucial role in damage induced by carvacrol and its mixture with thymol, as demonstrated by higher ROS levels and lower GSH levels. At low concentrations, both components play a protective role in Caco-2 cells against H_2O_2-induced damage (Llana-Ruiz-Cabello et al., 2015). Indeed research suggests that the cytotoxic effect of these components on eukaryotic cells consists in

induced apoptosis by the direct activation of the mitochondrial pathway (Bakkali et al., 2008; Yin et al., 2012). These components would affect inner cell membranes and organelles like mitochondria by provoking their permeabilization and depolarization. Changes in membrane fluidity may then result in the leakage of radicals, cytochrome c, calcium ions and proteins by acting as pro-oxidants. The intracellular redox potential and mitochondrial dysfunction would lead to cell death by apoptosis and necrosis (Bakkali et al., 2008).

Very few studies have investigated the mutagenic and genotoxic potential of carvacrol and thymol, but results are sometimes contradictory. Llana-Ruiz-Cabello et al. (2014) evaluated the potential mutagenic activity of the current usage concentrations of carvacrol and thymol by the bacterial reverse-mutation assay (Ames test), and their genotoxic activity using the comet assay on intestinal cell line Caco-2. These authors found that carvacrol exhibited mutagenic activity at 115-230 μM concentrations and genotoxic potential at a concentration of 460 μM. Thymol, on the contrary, showed no mutagenic or genotoxic effects at any tested concentration (0-250 μM). However, other works have reported no or low levels of genotoxicity and mutagenicity for carvacrol (Maisanaba et al., 2015; Ündeğer et al., 2009).

In vivo studies report adverse effects of acute and prolonged oral exposure to carvacrol and thymol in mice, rats and rabbits (Andersen, 2006). The LD_{50} for oral exposure to carvacrol and thymol in rats is 810 mg/kg bw and 980 mg/kg bw, respectively. For chronic exposure, no repeated dose toxicity data are available for carvacrol, while the thymol NOAEL value determined after subchronic exposure in rats is 667 mg/kg bw/day (ECHA, 2021).

Moreover, exposure to these compounds may cause allergic reactions in humans like dermatitis and skin inflammation (Salehi et al., 2018). Indeed carvacrol is classified as skin corrosive category 1B/C via acute inhalation and dermal exposure (ECHA, 2021).

196 **Table 1.** *In vitro* and *in vivo* toxicological effects described for carvacrol, thymol, eugenol and
197 vanillin.

EOC	Toxicological effects	References
Carvacrol	***In vitro***	
	Cytotoxic effects on Caco-2 cells	Llana-Ruiz-Cabello et al. (2014)
	Mutagenic effect on Caco-2 cells in the Ames test and genotoxic activity in the comet assay	Llana-Ruiz-Cabello et al. (2014)
	Weak genotoxic effects on mouse lymphoma cells in the micronucleus test	Maisanaba et al. (2015); Ündeğer et al. (2009)
	No genotoxic potential for Chinese hamster lung fibroblast (V79) cells in the comet assay	Ündeğer et al. (2009)
	In vivo	
	Acute toxic effects after oral exposure in mice, rats and rabbits. Skin irritation after acute dermal exposure in mice	Andersen (2006)
Thymol	***In vitro***	
	No cytotoxic effects (250 μM) but lipid degeneration, mitochondrial damage, nucleolar segregation and apoptosis	Llana-Ruiz-Cabello et al. (2014)
	No cytotoxic effects on peripheral blood mononuclear cells (100 μM)	Deb et al. (2011)
	Cytotoxic activity against V79, HepG2 and Caco-2 cells	Slamenová et al. (2007)
	No mutagenic or genotoxic effects at any tested concentration (0-250 μM)	Llana-Ruiz-Cabello et al. (2014)
	Genotoxic effects on V79 cells (25 μM) in the comet assay	Ündeğer et al. (2009)
	In vivo	
	Toxic effects after acute, short-term and prolonged oral exposure at high doses in *in vivo* studies	Andersen (2006)
	Allergic reactions in human	Salehi et al. (2018)
Eugenol	***In vitro***	
	Cytotoxicity in human HFF fibroblasts and HepG2 cells	Babich et al. (1993)
	Cytotoxic effects on the human osteoblastic (U2OS) cell line	Ho et al. (2006)
	Cytotoxic effects on human fibroblasts and endothelial cells	Prashar et al. (2006)
	Genotoxicity in V79 cells by the chromosomal aberrations test	Maralhas et al. (2006)
	Genotoxic effects on Chinese hamster ovary (AA8) cells	Martins et al. (2011)
	In vivo	
	Respiratory problems after exposure in rats.	Wright et al. (1995)
	Kidney and renal damage in frogs at anesthetic doses	Goulet et al. (2011)
	Genotoxic effects on *Drosophila melanogaster*	Munerato et al. (2005)
	Adverse reactions in humans (skin irritation, ulcer formation, dermatitis and slow healing)	Kamatou et al. (2012)
Vanillin	***In vitro***	
	Low *in vitro* toxic effects on murine macrophage cells	Oliveira et al. (2014)
	Cytotoxic effects on HepG2 cells at high concentrations	Fuentes et al. (2020)

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4. Eugenol

Eugenol (4-allyl-2-methoxyphenol) is a phenylpropene extracted from certain essential oils. It is the main component of clove oil and is also present in essential oils or extracts of many other plants, including cinnamon, basil and nutmeg (Kamatou et al., 2012). Eugenol is applied as a flavoring to food products, fragrances, cosmetics and personal care products (Nejad et al., 2017). In dentistry, it is widely used during the manufacture of dental plasters, fillings and cements for its analgesic and anti-inflammatory properties (Rojo et al., 2006). Other uses include anesthetic in aquaculture (Palić et al., 2006) and a substrate for vanillin production (Kaur & Chakraborty, 2013).

Eugenol has been well-studied for its antimicrobial properties in the food industry. Antimicrobial effects have been reported against a wide variety of foodborne and food spoilage bacteria, yeasts and fungi (De Souza et al., 2014; Tippayatum & Chonhenchob, 2007). Eugenol antimicrobial activity has been associated with the ability of its hydroxyl group to disrupt the cytoplasmic membrane and the cell wall, and to interact with proteins, to result in intracellular content leakage and the disruption of the proton motive force (Hyldgaard et al., 2012).

Besides its antimicrobial role, and its analgesic and anesthetic action, eugenol exhibits anti-oxidant and anti-inflammatory effects at low concentrations (Fujisawa et al., 2002). Eugenol has demonstrated to exert a beneficial action on both related properties through the inhibition of enzymes and oxidative processes (Barboza et al., 2018). Importantly, it has been found to inhibit lipid peroxidation at initial levels, by blocking secondary radicals derived from endoplasmic reticulum lipids (Nagababu et al., 2010). In line with this, the pharmacological properties of eugenol have been described for the treatment of diseases associated with oxidative stress and inflammatory responses. This compound exhibits a neuroprotective potential and offers hypolipidemic and anti-diabetic

effectiveness. Moreover, eugenol has demonstrated anti-cancer activity by inhibiting propagation of different cancer cell types, an anti-mutagenic potential against different genotoxic compounds, and its use in regenerative medicine has been proposed since the proliferation and migration promotion of stem cells *in vitro* has been demonstrated (Khalil et al., 2017; Sisakhtnezhad et al., 2018).

On the toxicological profile of eugenol, i*n vitro* studies have demonstrated its cytotoxic potential against different cell types in a dose-, frequency- and duration-dependent manner (Babich et al., 1993; Ho et al., 2006; Prashar et al., 2006) (Table 1). Intracellular glutathione depletion levels have been described as one of the mechanisms that underlie eugenol-induced cytotoxicity (Ho et al., 2006). This is because, despite its anti-oxidant activity at low concentrations, eugenol acts as a pro-oxidant agent at high concentrations, which enhances the generation of free radicals and results in tissue damage (Fujisawa et al., 2002). The *in vitro* genotoxic potential of eugenol has also been described. Maralhas et al. (2006) found that eugenol induces chromosomal aberrations and endoreduplication in V79 Chinese hamster fibroblasts in a concentration-dependent manner in the absence of an exogenous biotransformation system, suggesting a direct genotoxic mechanism, possibly acting as a topoisomerase II inhibitor. Similarly, Martins et al. (2011) found that a 1-hour exposure to eugenol produces both DNA single strand and double strand breaks in Chinese hamster ovary (CHO-K1) cells, and apoptosis was also observed after a 24-hour incubation period to the 750 μM concentration. Those effects were related to oxidative damage caused by ROS production.

Eugenol is considered not acutely toxic and has an LD_{50} value over 2,000 mg/kg bw for rats, and between 1,500 and 3,000 mg/kg bw for mice, while chronic studies establish a NOAEL value of 300 mg/kg bw/day (ECHA, 2021). However, acute *in vivo* studies found that eugenol causes respiratory distress with hemorrhagic pulmonary edema after

injection in rats (Wright et al., 1995), kidney damage, apoptosis and morphological alteration in renal cells of exposed frogs at aesthetic doses (Goulet et al., 2011), and genotoxic effects on *Drosophila melanogaster* (Munerato et al., 2005).

In humans, the use of eugenol in fragrance ingredients and dental products has been associated with different adverse reactions, including skin irritation, ulcer formation, dermatitis and slow healing. A case study has also revealed adverse side effects after unintentional ingestion of eugenol that results in similar hepatotoxic effects to paracetamol poisoning (Kamatou et al., 2012).

5. Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a phenolic aldehyde and the main component of the extract of the bean and pod of the vanilla orchid. It is one of the most widely used flavor compounds in foods, pharmaceuticals, fragrances and personal care products (Al-Naqeb et al., 2010). In the food industry, it is often employed in processed foods as a flavoring agent, and as a sweetener in dairy, bakery and confectionary products, and also in beverages. Vanillin is also used in aromatherapy and is an ingredient of perfumes, toothpastes, soaps, cosmetics, and other personal and household products. In the chemical and pharmaceutical industry, vanillin is involved in the manufacture of herbicides, antifoaming agents or drugs like L-dopa. Other products that may also contain vanillin include cigarettes, cattle feed or pharmaceuticals, paints and plastics where it is used as an odor-masking agent (Cheng et al., 2007).

Albeit less studied than carvacrol, thymol or eugenol, the antimicrobial action of vanillin has also been demonstrated *in vitro* against different food-related bacteria, yeasts and molds (Hyldgaard et al., 2012). Vanillin's antimicrobial mode of action has not been

completely elucidated, but it has a demonstrated deleterious effect on cytoplasmic membrane integrity, with the resulting loss of pH homeostasis and respiratory activity inhibition (Fitzgerald et al., 2004).

The antioxidant capacity of vanillin has been also demonstrated, for instance, by protecting against the oxidative damage induced by photosensitization (Kamat et al., 2000). This antioxidant action has been related to the ROS-scavenging ability or the modulation of hepatic enzyme antioxidants such as catalase and SOD (Makni et al., 2011; Tai et al., 2011). Besides, vanillin and its analogues have also shown other beneficial properties, such as antimutagenic (Lee et al., 2014), anticarcinogenic (Bezerra et al., 2016; K. Ho et al., 2009) and hypolipidemic activity (Al-Naqeb et al., 2010).

The toxicological effects of vanillin are reported less than those of other EOCs. It is considered to have a low cytotoxic potential as only high concentrations (mM range) reduce cell viability in a concentration- and time-dependent manner (Oliveira et al. 2014; Fuentes et al. 2020). Additionally, vanillin is not considered harmful by ingestion, with an LD_{50} of 3978 mg/kg bw for acute oral exposure and a NOAEL value of 650 mg/kg/day, as determined by a subchronic study in rats (ECHA, 2021).

6. Conclusions

The use of essential oils and their main components has considerably increased in recent years and the market is predicted to grow because of rising consumer demand for natural products and their potential use in multiple applications. Therefore, prolonged consumer exposure to these compounds is expected in the foreseeable future. Carvacrol, thymol, eugenol and vanillin are four of the most used and investigated EOCs for their relevant biological properties. Different studies describe adverse effects after exposure to medium

and high concentrations of these components, although information remains limited. Thus, more toxicological research, including chronic exposure studies and combined exposures to different components, is necessary to not only elucidate the possible risks deriving from increased exposure to these components, but to also guarantee their safety for human health and the environment.

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Disclosure statement

The authors report no conflict of interest.

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