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Qualitative and quantitative phytochemical screening of Vitex negundo L. extract using chromatographic and spectroscopic studies

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Abstract

In the present study, the plant was identified as Vitex negundo (L) and assigned a voucher specimen accession number as SJCBOT2701. The shade dried leaf powder was extracted with water, and organic solvents such as ethanol, and ethyl acetate. The metabolic extractive value was comparatively higher in the aqueous extract (11.2%) when compared to ethanol (8.6%) and ethyl acetate (6.7%) extracts. Qualitative analysis of extracts revealed the presence of alkaloids, flavonoids, steroids, tannins, coumarins, phenols and terpenoids, and quantified for its phytochemical constituents with High performance Thin Layer Chromatography, Liquid Chromatography- tandem Mass Spectrometry and Gas Chromatography Mass Spectrometry. The results revealed the presence of dodecanoic acid, coumarine-3-carbohydrazine, tetradecanoic acid, trihydroxy isoflavone, hexadecanoic acid, 12-octadecenoic acid, 11-heneicosanone and high amount of flavonoids such as chalcones, flavones, flavonols and isoflavones. The presence of flavonoids and other secondary metabolites may be attributed to the pharmacological activities of V. negundo. Comparison of results with other reports revealed that the secondary metabolites present in the shrub varies from place to place and environment to environment which might be due to the environmental and ecological factors.

Keywords: Vitex negundo, Phytochemicals, Aqueous extract

Introduction

Plants have great potential in producing new drugs that can be used to treat chronic and infectious diseases. Since ancient times, every culture throughout the world has been using herbal and natural products for the benefit of human and animals. Several bioactive chemicals are found in the

medicinal plants which play important role in the field of drug discovery. According to a report of World Health Organization, more than 80% of world's population depend on traditional medicine for their primary health care needs (Duraipandiyan et al., 2006). The increased interest in plant derived drugs is mainly because of the wide spread belief that 'herbal medicine' is safer and cost effective than synthetic drugs. It is therefore essential for systematic evaluation of plants used in traditional medicine for the treatment of various ailments.

Vitex negundo L. (Verbenaceae) commonly known as five-leaved tree, grows gregariously in wastelands and is also planted as a hedge-plant in many parts of India (Gupta et al., 2005: Watt, 2014.). The plant parts such as leaves, bark, fruits, roots and seeds are used in treatment of several diseases. V. negundo has been used in traditional medicine (Chandramu et al., 2003) for the treatment of eye-disease, toothache, inflammation, leucoderma, enlargement of the spleen, skin-ulcers, in catarrhal fever, rheumatoid arthritis, gonorrhoea, and bronchitis. It is also used as tonic, vermifuge, lactagogue, emmenagogue, antibacterial, antipyretic and antihistaminic agent. Aqueous extract of mature fresh leaves exhibited anti-inflammatory, analgesic and antihistamine properties (Dharmasiri et al., 2003). Leaves of V. negundo have been shown to have mosquito repellent property (Hebbalkar et al., 1992) antiulcerogenic, antiparasitic (Parveen, 1991), antimicrobial (Rusia and Srivastava, 1988), and hepato-protective properties (Prajapati et al., 2003). The extract of V. negundo was found to contain many polyphenolic compounds, terpenoids, glycosidiciridoids, flavonoids, and alkaloids. Even though, many reports are available on the medicinal importance of V. negundo, reports on phytochemical properties of this therapeutically important shrub in this geographical location is meagre. In the present study, shade dried leaf powder was extracted with water, and organic solvents such as ethanol, and ethyl acetate, further qualitatively and quantitatively screened for its phytochemical contents using High Performance Thin Layer Chromatography (HPTLC), Liquid Chromatography-tandem Mass Spectroscopy (LC-MS) and Gas Chromatography-Mass Spectroscopy (GC-MS) analysis.

Materials and Methods

Collection and identification of plants

Fresh leaves of V. negundo were collected from Sooriyur village, Tiruchirappalli District, Tamil Nadu. The identification and taxonomic authentication of the plant material was done by comparing with the voucher specimens from The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College (Autonomous), Tiruchirapalli, Tamil Nadu. The collected leaves were cleaned and

washed thoroughly, shade dried at room temperature and pulverized to make a coarse powder. The powder was packed in zip lock covers, labelled and stored in air tight container.

Extraction of Vitex negundo leaf extracts

The aqueous and ethanolic extracts of V. negundo leaf powder (30g) were prepared by soxhlet apparatus method with deionised water (170mL) and ethanol, respectively (Danlami et al., 2015). The ethyl acetate extract was prepared by direct extraction method with 30g of leaf powder submerged in 170mL of ethyl acetate with constant stirring for 24 hours (Pinelo et al., 2007). After extraction, the extract was filtered through Whatmann No.1 filter paper and condensed using rotary vacuum evaporator. The weight of dried extracts was measured and dissolved in the respective solvent to attain final concentration of 5mg mL^{-1}. Extractive values of the aqueous, ethanolic and ethyl acetate extracts were calculated using the following formula:

$$\text{Extractive Yield (\%)} = \frac{\text{Extract obtained (g)} \times 100}{\text{Plant material (g)}}$$

Qualitative analysis of phytochemicals

The phytochemicals present in the leaf extracts of V. negundo were qualitatively evaluated as per the method reported by Santhi and Sengottuvel (2016). Presence of alkaloids were evaluated by Mayer's test, Wagner's test and Dragendroff's test. Presence of saponins were evaluated by foam test. Phytosterols were analysed by Salkowski's test. Phenols and tannins were detected by Ferric chloride test. Flavonoids were detected by Lead acetate test and alkaline reagent test. Coumarins were analysed using Sodium hydroxide. Proteins and amino acids were detected by Xanthoproteic test.

Quantitative analysis of phytochemicals

The aqueous extract of V. negundo was subjected to phytochemical screening using HPTLC fingerprint analysis, LC-MS and GC-MS analysis to evaluate the presence of various secondary metabolites demonstrated in qualitative phytochemical screening.

HPTLC fingerprint analysis

The plant extract was subjected to phytochemical screening by HPTLC fingerprint analysis following standard methods. The plant extract was applied on the TLC aluminium plate precoated with silica gel 60 F_{254} using Camag's ATS4 applicator and developed by gradient mode using the automated multiple developer (AMD2). The sample (15 µL) was applied on the plate with 6 mm band width

fitted with a micro syringe using CAMAG HPTLC Linomat IV sample applicator. The plate was developed in the mobile phase using ethyl acetate and n-butanol (6:4 ratio) up to 85 mm in a twin trough CAMAG glass chamber previously saturated with mobile phase for 20 minutes at 25°C. After development, the plate was air dried and photo documented using Camag's TLC Visualizer under 254 nm (D2 lamp, Absorption mode) and 366 nm (Hg lamp, Fluorescence mode) and the finger print profiles were recorded. The plate was further derivatized using vanillin-sulphuric acid reagent and heated at 105°C and the coloured spots were recorded at 520 nm. The Rf values and fingerprint data were recorded and the results were calculated by densitometric evaluation of each chromatogram.

LC-MS analysis

The initial separation was performed on Ultra High-Performance Liquid Chromatography (UHPLC) system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu UHPLC: Nexera UHPLC system Column: Shim-pack XR-ODS III (100 × 2 mm, 2.2 µm particle size). The column temperature was at 40°C. The mobile phase consisted of (A) acidified water (0.1% formic acid v/v) and (B) Acetonitrile. The column was re-equilibrated for 5 min using the initial solvent composition. Flow rate was set to 1 mL min^{-1}. The samples were kept in amber vials at 4°C in the auto-sampler and the injected volume was 5 µL. The separation was performed at 25.0 ± 0.1°C under following conditions. Ionization: ESI (Positive/Negative), Ion spray voltage at +4.5 kV/-3.5 kV. The Dwell time versus Pause time was 5 ms / 1 ms. Ambient CDL temperature: 250°C. Block Temperature: 400°C. Detector voltage: 1.3 kv. Nebulizer gas flow: 1.5 L min^{-1}. Drying gas: 10 L min^{-1} (Chen et al., 2013).

GC-MS analysis

The GC-MS analysis of aqueous leaf extract of V. negundo was done using GC-7890A/MS-5975C (Agilent Technologies, Santra Clara, CA, USA) equipped with HP-5MS column 30 m length × 0.25 mm diameter × 0.25 µm thickness with single quadrupole detection system. Helium (99.99%) was used as the carrier gas with a flow rate of 1.0 mL min^{-1}. Oven temperature and injection temperatures were maintained at 280°C. Injection volume was set at 10 µL. The quantitative estimation of the chemical constituents present in the leaf extract of V. negundo was expressed as percentage based on peak area formed in the chromatogram. The compounds were identified by comparing the data with the existing spectrum of National Institute of Standard and Technology (NIST) library database.

RESULTS AND DISCUSSION

Identification of plant and assigning specimen accession number

The identification and taxonomic authentication of the plant material was done by comparing with the voucher specimens from 'The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College (Autonomous), Tiruchirapalli 620 002, Tamil Nadu'. The plant was identified as Vitex negundo (L). It was assigned a voucher specimen accession number SJCBOT2701.

Extractive value of the crude extracts

Maximum extractive percentage was found in the aqueous leaf extract of V. negundo (11.2%) followed by ethanolic leaf extract (8.6%). The least extractive percentage was found in ethyl acetate extract (6.7%). In a similar study, the maximum soluble extractive percentage of V. negundo leaves were found to be 6.75% in aqueous extract, 4.35% in methanolic extract and 1.8% in acetone extract (Pawar and Kamble, 2017). Bagde et al., (2019) also reported that the alcohol extractive value of V. negundo leaf powder was 9.17%. It has been earlier reported that hot solvent extraction methods are comparatively better than non-thermal methods for extract preparation from V. negundo leaves (Abidin et al., 2014). The differences in the extractive yield for the different solvents may be due to the differences in the polarity of the solvent used, solubility of the metabolites present in the sample, the availability of extractable components in the sample and different extraction techniques.

Qualitative analysis of phytochemicals present in V. negundo leaf extract

Plants are rich sources of bioactive chemical compounds, pigments, steroids etc. In general, plants are able to synthesize a variety of chemical substances such as non-protein amino acids, alkaloids, terpenes, flavonoids and their chemical diversity has increased greatly during the course of evolution along the periodical climate changes and insect feeding pressure. One of the features of secondary metabolism is to cope with and adapt to a continually changing environment relates to chemical diversification, with intra-population variation being inherent. The results of qualitative phytochemical analysis are tabulated in Table 1.

Table 1: Phytochemical screening methods - Qualitative analysis of phytochemicals in aqueous, ethanolic and ethyl acetate leaf extracts

S. No	Phytochemicals	Test	Aqueous	Ethanol	Ethyl acetate
1	Alkaloids	Wagner's test	Present	Present	Present
2	Flavonoids	Lead acetate test	Present	Present	Present
3	Steroids	Salkowski's test	Present	Present	Present

4	Tannins	Ferric chloride test	Present	Present	Present
5	Saponins	Foam test	Absent	Absent	Present
6	Proteins	Xanthoproteic test	Absent	Present	Absent
7	Coumarins	Sodium hydroxide	Present	Present	Present
8	Phenols	Lead acetate test	Present	Present	Present
9	Terpenoids	Salkowski's test	Present	Present	Present

It has been recognized that phenolics and flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The qualitative phytochemical analysis revealed that the alkaloids, flavonoids, steroids, tannins, coumarins, phenols and terpenoids were present commonly in all the three extracts. The presence of various bioactive secondary metabolites in the plant might be responsible for its numerous pharmacological properties. In a similar study, the aqueous leaf extract was found to contain carbohydrates, flavonoids, glycosides, proteins and coumarins (Abidin et al., 2014). Sahayaraj and Ravi (2008) also found that, aqueous extract contains carbohydrates, phenolic compounds, saponins, xanthoproteins, tannins and flavonoids. The ethanolic leaf extract contained all major phytochemical constituents including alkaloids, flavonoids, tannins, terpenoids, glycosides, phenols and steroids except carbohydrates (Arokiyaraj et al., 2009). Chitra et al., (2009) also reported the presence of glycosides, alkaloids, lignin, flavonoids and saponins in the ethanolic leaf extract of V. negundo.

Quantitative analysis of phytochemicals

Currently, the chromatographic fingerprint technique was effectively used as a tool to evaluate the presence of secondary metabolites and their derived products in the plant extracts. The chromatographic fingerprints established by TLC, HPLC, HPTLC, GC-MS and LC-MS have been recognized as rapid and reliable means for the identification and quantification of herbal medicines. In Indian traditional medicine and ethnic medical practices, aqueous extracts of the medicinal plants and herbs have been generally used to make medicinal remedies for the treatment of various ailments. Hence in the present study, the phytochemicals and active secondary metabolites present in the aqueous leaf extract of V. negundo was estimated quantitatively by HPTLC fingerprint, LC-MS and GC-MS analysis.

HPTLC finger print analysis

The HPTLC finger print profile of V. negundo leaf extract confirms the presence of numerous phytochemicals as bands under UV (254 nm and 366 nm) visualization along with suitable R_f values

(Figure 1). Under 254 nm, three bands at Rf values of 0.74 (dark green), 0.63 (dark green) and 0.11 (green) were observed. Under 366 nm, two bands at Rf values of 0.74 (blue) and 0.24 (dark blue) were recorded. After derivatization with Vanillin-Sulphuric acid under visible light, four bands were observed with Rf values of 0.82 (pink), 0.74 (dark pink), 0.62 (brown) and 0.28 (pink). The different colour bands present in the HPTLC fingerprint revealed the presence of different Phyto-constituents in the aqueous extract of V. negundo.

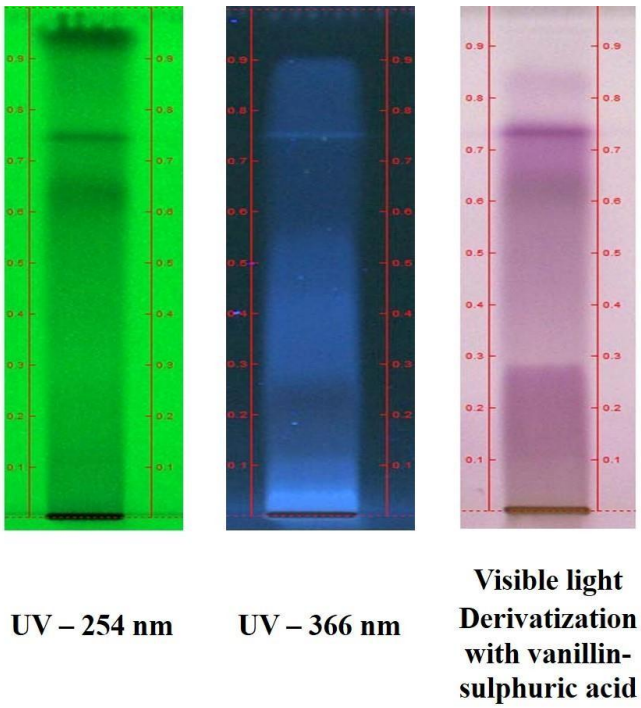

Figure 1: Quantitative analysis of phytochemicals by TLC fingerprint analysis

The results were vell correlated with the previous studies where, Yadav et al., (2012) also studied the TLC fingerprint profile of V. negundo leaf petroleum ether extract. They reported the presence of different types of phytochemicals with the Rf values of 0.61, 0.56, 0.54, 0.47, and 0.38. In a similar study, the phytochemical contents of V. negundo leaf extract was evaluated with TLC fingerprint analysis where, the Rf values were recorded as 0.61, 0.33, and 0.25 (Gautam et al., 2008). In another study, TLC fingerprint analysis of V. negundo L. leaf extract revealed the presence of three spots in the visible region with Rf values of 0.84, 0.77, 0.32, five spots in the 254 nm visualization having Rf values of 0.84, 0.77, 0.72, 0.62, 0.32 and four spots in the 366 nm visualization with Rf values of 0.84, 0.77, 0.62, and 0.32 (Dwivedi et al., 2021).

LC-MS analysis

LC-MS is widely applied in the fields of proteomics, pharmacokinetics and drug discovery because of its quick molecular separation and structure identification (Pitt, 2009). It proves effective in separating natural compounds which are thermo labile in nature. The LC-MS chromatogram in positive and negative ionization modes revealed the presence of important flavonoids in V. negundo leaf extract. The MS spectrum in positive ionization mode depicted 101 peaks with base peak value at 381.10 m/z, while in negative ionization mode there were 41 peaks with base peak value at 190.95 m/z. The peaks shown in the MS spectrum represent flavonoids which may include chalcones, flavones, flavonols and isoflavones. They play pivotal role as signal molecules, detoxifying agents, anticancer compounds and antimicrobial defensive compounds (Panche et al., 2016). Similarly, Nadeem et al., (2020) evaluated the phytochemical constituents present in the ethanolic leaf extract of V. negundo by LC-MS/MS analysis and revealed the presence of 15 bioactive molecules including flavanone, agnuside, casticin, herbacetin rhamnoside, kaempferol, luteolin-7-glucoside, negundoside, p-hydroxybenzoic acid, protocatechuic acid, quinic acid, vitedoin A, and vitexin.

In another study, presence of 17 bioactive flavonoids and phenolics including Aucubinb, p-Hydroxybenzoic acid, Chlorogenic acid, Schaftoside, Agnuside, Isoschaftoside, Vitexin, Isovitexin, Hyperoside, Luteoloside, Kaempferol-3-Orutinoside, Isochlorogenic acid, Isochlorogenic acid A, Isochlorogenic acid C, Quercetin, Apigenin, Casticin were reported in the ethanolic leaf extract (Huang et al., 2015). In a similar study, the presence of 39 compounds in positive ion mode and 34 compounds in negative ion mode were found belonging to Iridoid glycoside, Terpene, Polyphenol, Hydroxyindoles, Diarylethene, Flavonoid, Flavonols, Glycosyloxy flavone, Quinic acid, Triterpenoid, Diterpenoid, Lignans, and fatty acids (Dwivedi et al., 2021). The results conclude that the presence of flavonoids depicted by the peaks in the LC-MS spectral analysis may be attributed to the pharmacological activities of V. negundo.

GC-MS analysis

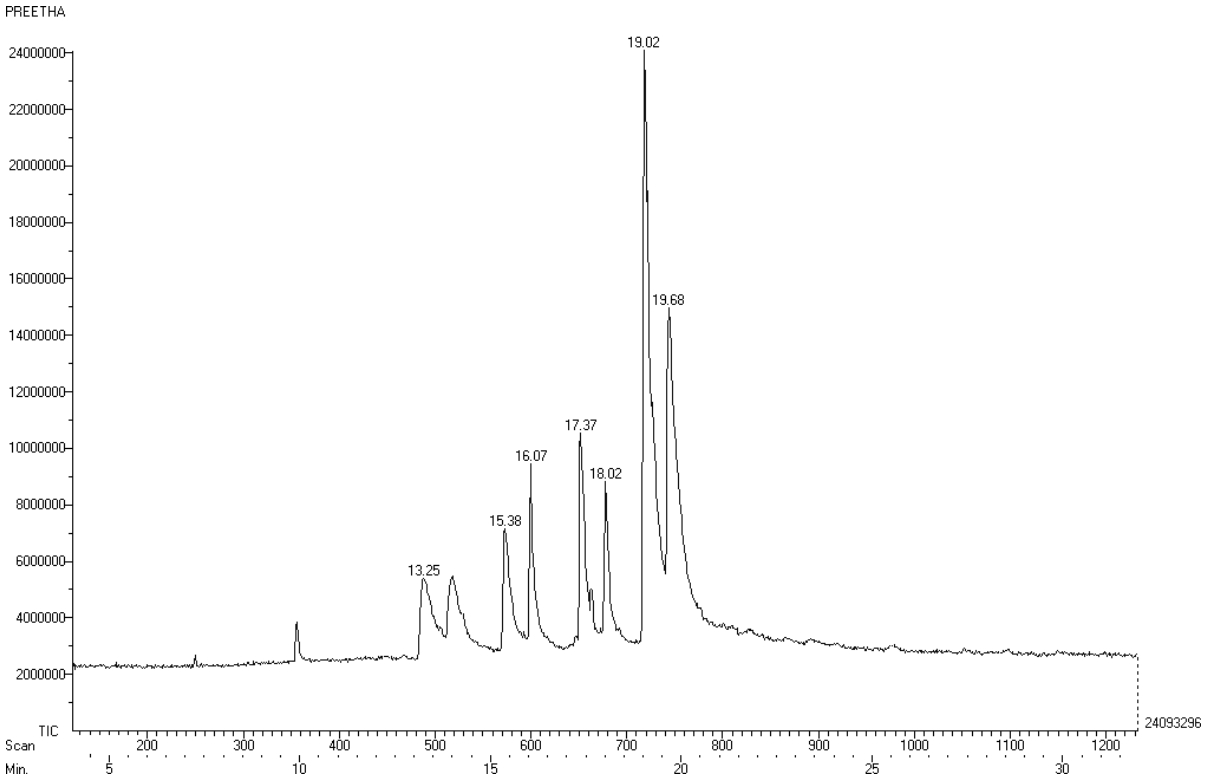

Figure 2: GC-MS spectrum of V. negundo leaf aqueous extract

The GC-MS analysis of V. negundo leaf extract revealed the presence of secondary metabolites responsible for the pharmacological activity of the plant (Figure 2). The major components were found to be dodecanoic acid (RT:13.25), coumarine-3-carbohydrazine (RT:15.38), tetradecanoic acid (RT:16.07), trihydroxyisoflavone (RT:17.37), hexadecanoic acid (RT:18.02), 12-octadecenoic acid (RT:19.02), 11-heneicosanone (RT:19.68). The spectrum of unknown components obtained in the GC-MS analysis were compared with the spectrum of known components stored in the NIST library, by analysing the retention time, molecular weight, molecular formula and composition percentage in the sample material. Similarly, Sahayaraj and Ravi (2008) reported the presence of 1H-indene, cyclododecanol, 1,2-dihexylcyclopropene-3- carboxylic acid, 2-heptenoic acid, trans-caryophyllene, cyclohexane, farnesol, pentadecane and 1-octanol from the benzene and chloroform extract of V. negundo leaves. Simultaneously, Kumar et al., (2010) revealed the presence of Benzoic acid 3-hydroxy, Ledol, 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-, Vitamin E, 4HPyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, Caryophyllene, and n-Hexadecanoic acid which might contribute to the medicinal property of the plant. Other compounds reported in the leaf extract include Tetramethoxyflavone, trimethoxyflavone, ascerosin, 5-glucosylrhamnoside; casticin, lutcolin, chrysoplenol D, isooxientin, p-hydroxybenzoic acid (Dayrit and Lagurin, 1994); iridoids, sabinene, p-cymene, ß-phelladune, γ-terpinene, terpinen-4-ol, ß–caryophyllene, viridiflorol (Mallavarapu et al.,

1994); mono and sesquiterpenes (Jirovetz et al., 1998). The results clearly depict the presence of several important secondary metabolites including flavonoids in the aqueous leaf extract of V. negundo. Comparison of results with other available reports (Kumar et al., 2010; Sahare et al., 2008, Dhakal et al., 2009) revealed that the secondary metabolites present in the shrub varies from place to place and environment to environment. This might be due to the influence of genetics, environmental and ecological factors including latitude, longitude, rainfall, climate change, soil microbiome, temperature, soil nutrients, endophytic organisms, soil nature, post-harvest storage, processing, phyto-pathogens and cultural practices.

The qualitative analysis of the aqueous, ethanolic and ethyl acetate leaf extract showed the presence of important alkaloids, flavonoids, glycosides, phenols, terpenoids and coumarins. The quantitative analysis of the aqueous extract of V. negundo using HPTLC fingerprint analysis, LC-MS/MS and GC-MS analysis confirmed the presence of flavonoids and other phytochemicals. The unidentified peaks represented in the chromatogram indicate the presence of variety of important secondary metabolites which may be responsible for the multiple beneficial effects of the plant. Comparison of results with other reports about the phytochemical content of V. negundo revealed that the secondary metabolites present in the shrub varies from place to place which might be due to the environmental and ecological factors.

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Conflict of interest

The authors declare that they have no conflict of interest.

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