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Bioactivity of Leaves, Skins and Seeds of Berry Color Variant Grapevines (*Vitis vinifera* L.)

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Research Article

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ABSTRACT

Grapevine synthesizes a wide variety of polyphenolic compounds, which can be beneficial both the plant and human health, due to their antioxidant effects. In this study, total antioxidant capacities (TAC), flavonoid and flavanol content in various plant parts of berry color variants (*V. vinifera L.* conc. Gohér) were evaluated for the first time. The current research aimed to examine the effect of sunlight exposure on the accumulation of polyphenolic compounds in shaded and sun exposed leaves and also included the measurements of berry skins and seeds. Two electron transfer based techniques were used for antioxidant analyses; Trolox Equivalent Antioxidant Capacity (TEAC) and Ferric Reducing Antioxidant Potential (FRAP). In addition, Total Reducing Capacity (TRC) and relative amounts of compound groups such as flavonols and flavanols were measured using photometric assays. Our results revealed significant differences in the studied parameters among the varieties and their different tissues. Furthermore, we documented different sunlight acclimation of leaves in terms of differently increased TAC values and flavonoid content. Our study proved that grapevine leaves contain more antioxidants than berry skins and they have more varied antioxidant composition than seeds, therefore this plant part is an excellent source of bioactive compounds, applicable for food, chemical and pharmaceutical industries.

INTRODUCTION

Polyphenols are a large family of secondary metabolites found in all tissues and organs of the plants. They include flavonoids like flavonols, anthocyanins, flavanols, and non-flavonoids like phenolic acids and stilbens [1]. Most of them, mainly flavonoids, outperform well-known antioxidants, because of their strong capacity to donate electrons or hydrogen atoms [2]. A number of studies have shown that the health protective effects of fruit, vegetables and beverages might be associated with their polyphenolic content [3,4]. Grapevine is a polyphenol-rich plant, hence consumption of its berry, juice or wine may serve health-promoting benefits. Several studies were devoted to determine the polyphenolic compounds in berry skins and seeds of grape [5-9]. Since *Vitis vinifera* leaves are also rich in polyphenols, they provide an excellent source of bioactive compounds applicable for health protection [10,11]. Nevertheless, most of the grapevine leaves are only the waste of the table grape and wine production in many regions of the world.

Several researches studying quantity and quality of polyphenolic content in different parts of grapevine focused on variation among varieties [12-14], however different biotic and abiotic factors influence significantly these parameters [15-17]. Flavonoids are involved in an array of defense processes, but primarily they play important antioxidant role in the photoprotection [18,19]. Therefore, bioactivity of grapevine leaves is highly influenced also by the natural light conditions of the canopy.

In our study, we evaluated experimental data that suggest that grapevine leaves, mainly the sun acclimated ones, are rich source of natural antioxidants. Comparison of antioxidant capacities, flavonoid and flavanol content in different tissues of three berry color variant grapevines was performed to determine the influence of variety on the measured parameters and to study the bioactivity of different plant parts. In addition, we intended to examine the effect of natural sunlight-exposure on the

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above mentioned values of leaves. The studied grapevine varieties offer a suitable model to determine the importance of genetic background and the natural sunlight conditions influencing grapevine leaf antioxidant properties attributed mainly to polyphenolic content.

METHODOLOGY

Plant material and sample preparation

Gohér conculta is important part of our viticultural heritage, highly adapted to local environmental conditions. The members of the conculta – G. White, G. Altering, G. Red – are genetically closely related, but their genetic background differs mainly in the phenylpropanoid pathway, resulting berry color variant phenotypes [20]. Therefore they provide a suitable model to study the interaction between genetic and environmental factors in determination of grapevine phenolic composition and antioxidant capacity. The berries of G. Altering change color during ripening; they are light purple at veraison and white at harvest time.

Healthy, mature leaves of Gohér varieties were harvested from the shady core and direct sunshine exposed parts of the canopy during sunny days of the first week of August 2016, in the autochthonous grapevine collection of the Research Institute for Viticulture and Oenology (University of Pécs). The experimental vineyard consisted of five-ten plants of each variety, all of them grown under the same climatic conditions and agronomic practices. The varieties are grafted on Teleki 5C (V. berlandieri x V. riparia) rootstock in mid-high cordon vertical training system.

The sun exposed leaves were harvested at 10:00–11:00 a.m. from the eastern part of the canopy, where leaves received full sunlight during morning time until noon. Photosynthetically Active Radiation (PAR) conditions of sun and shaded leaves were 1800–2000 µmol photons m−2 s−1 and 500–600 µmol photons m−2 s−1, respectively. PAR was measured using a Cole Parmer radiometer (Cole-Parmer Instrument Co. Ltd., London, UK). Ripe berries were collected at the grape harvest time, during the last week of September 2016. Berries were manually skinned, seeds were separated from the pulp.

Three leaf extracts per variety were made of three sets of leaf samples (6-6 leaves each) collected at the same time from different plants. Berry samples were composed of 60 berries of 6 clusters from 3 plants per variety. Three biological replicates per tissue type per variety were performed for each parameter.

All samples were air dried in the dark at room temperature and grinded. 200 mg of pulverized leaves, skins and seeds were extracted with a mixture of ethanol and water (3:7 v/v) for photometric analyses, using ultrasonic bath for 40 mins. Purification of extracts was done by centrifugation and filtration through a 0.45 µm pore size Syringeless filter (Mini-Uniprep, Whatman). Samples were stored in the dark at 4°C until the analysis was carried out.

Photometric methods

The antioxidant effectiveness of the extracts was carried out using two different free radical-scavenging assays, TEAC and FRAP, due to their different sensitivity to various antioxidant compounds [21]. TAC (Total Antioxidant Capacity) values were measured as TEAC (Trolox Equivalent Antioxidant Capacity) using ABTS radical, following the method described by Re et al. (1999) [22]. FRAP (Ferric Reducing Antioxidant Power) assay was carried out according to Szőllősi and Szőllősi-Varga (2002) [23]. TRC (Total Reducing Capacity) was determined by Folin-Ciocalteu method [24]. Measurements of total flavonoid content by AlCl3 method and total flavanols by pDMACA method were specified and the protocols were optimised for plant samples as detailed in Csepregi et al. (2013) [25]. Antioxidant reactions, absorption of flavonoids and flavanols were detected with a Shimadzu UV-1800 spectrophotometer. Results were expressed as µM Trolox equivalents (µM Trolox) 100 mg^{-1} for TEAC, µM ascorbate equivalents (µM ASE) 100 mg^{-1} for FRAP, mg gallic acid equivalents (GAE) g^{-1} for TRC, mg quercetin equivalents (QE) g^{-1} for flavonoids and mg catechin equivalents (CAT) g^{-1} for flavanols of leaf, berry skin and seed dry weight.

Statistical analysis

Linear model analyses were carried out in R version 3.1.2 [26]. Dependent variables were the measured attributes (TEAC, FRAP, TRC, Flavonoids, Flavanols), while the independent variables were the plant organs and variety. All attributes were analyzed separately using linear model (function lm; Gaussian error distribution; link function: linear). Omnibus statistics of model were carried out with Type III F tests. Transformation and testing residuals were based on graphical evaluation according to Crawley (2014) [27]. For pair-wise comparisons, Dunnett post-hoc tests were conducted in both cases with multcomp-package [28]. Average values and standard deviation (SD) data were calculated using Microsoft Excel 2010 software. PCA analysis with biplot of all measured chemical compounds of the investigated Vitis samples was carried out in Past version 3.13 [29]. The scatterplot is based on an Euclidean distance measure of the original data points. Eigenvalues of axes were calculated with the SVD algorithm. To describe the relationship among TEAC, FRAP and TRC, Pearson's rank Correlation was used in R.

RESULTS AND DISCUSSION

Antioxidant activity and phenolic composition of sun exposed and shaded leaves

Antioxidant activities, flavonoid and flavanol contents of grapevine leaves are shown in **Table 1**. Antioxidant activities of

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sun exposed leaves of G. Altering were significantly higher than those of the other two varieties studied. According to our current knowledge, the parameters of a variety, which changes berry color during ripening, were analysed for the first time in this study. Significant differences were observed among the shaded leaves of the varieties, from which G. White produced the highest values.

Table 1 Antioxidant Activities, Total Reducing Capacity, Flavonoid and Flavanol Content of Sun Exposed and Shaded Leaves of Gohér Varieties (Mean ± Standard Deviation). Minuscules Represent Groupings for Each Measured Parameter, Based On Dunnett Post-Hoc Tests.

Leaf samples		TEAC (μM TE/ 100 mg dw)	FRAP (μM ASE/ 100 mg dw)	TRC (mg GAE/g dw)	Flavonoids (mg QE/g dw)	Flavanols (mg CAT/g dw)
G. White	Sun	154.4 ± 5.04^{a}	136.2 ± 6.46^{a}	202.3 ± 11.1^{a}	85.6 ± 1.45^{a}	20.3 ± 0.20^{a}
	Shade	146.7 ± 1.99^{a}	119.7 ± 7.57^{b}	179.0 ± 4.97^{a}	73.6 ± 1.92^{b}	19.2 ± 0.74^{a}
G. Altering	Sun	188.2 ± 2.09^{b}	168.7 ± 2.11^{c}	261.9 ± 17.05^{b}	95.9 ± 4.17^{c}	27.0 ± 0.58^{b}
	Shade	108.8 ± 4.05^{c}	88.9 ± 3.02^{d}	133.6 ± 2.45^{c}	58.3 ± 1.03^{d}	12.5 ± 0.40^{c}
G. Red	Sun	158.2 ± 5.23^{a}	129.0 ± 1.08^{a}	198.1 ± 11.68^{a}	73.9 ± 3.67^{b}	19.8 ± 1.00^{a}
	Shade	64.6 ± 2.02^{d}	53.0 ± 2.09^{e}	77.4 ± 5.64^{d}	31.3 ± 1.74^{e}	6.4 ± 0.27^{d}
In the same column mean values with different letters differ significantly ($p<0.05$)						

Total Reducing Capacity (TRC) of the extracts was analysed by the widely applied Folin-Ciocalteu (FC) method, which assay formerly intended to analyse total polyphenolic content, but FC reagent is nonspecific to polyphenolic compounds [21]. However, the simple and reproducible method can be used for antioxidant characterization, giving support to other antioxidant assays [30,31]. TRC varied between 77 mg GAE/g (G. Red shaded leaves) and 262 mg GAE/g (G. Altering sun leaves). Our results are in accordance to other white and red grapevine varieties [14,15], while Fernandes et al. (2013) [13] reported higher values of several Portuguese varieties (323-550 mg GAE/g). Sun acclimated leaves of G. White gave similar parameters compared to those of G. Red, while the results of shaded leaves of G. White were significantly higher than those of G. Red. The first observation supported Fernandes et al.'s (2013) [13] study, where the average TRC value of white varieties was slightly higher than that of red varieties. These significant differences supported the general observation, that white grapevine leaves display higher antioxidant capacities than red varieties [14].

In addition to the above mentioned antioxidant measurements, we analyzed total flavonoid and total flavanol content. The quantity of these polyphenolic groups, showed similar trend among varieties like antioxidant potentials. The amount of flavonoids and flavanols were the lowest in G. Red shaded leaves, while G. Altering sun leaves contained the highest quantities. Total flavonoid content varied between 31 mg QE/g (G. Red shaded leaves) and 96 mg QE/g (G. Altering sun leaves). The results of the sun exposed leaves are in accordance to those of Portuguese white and red grapevine leaves [14] and the results of sun and shaded leaves are above those presented by Farhadi et al. (2016) [12]. Flavanol content gave about 10-fold lower values than TRC (named as total phenolic content) according to Balik et al.'s (2008) [32] study in *V. vinifera L. varieties*. Amount of flavanons was about 4-fold lower than that of flavonoids in the leaves. Moreover, since the AlCl3-method, which quantifies total flavonoids, has higher sensitivity to the flavanon catechin than to the flavonol quercetin [25], our results proved the observation, that grapevine leaves are rich in non-flavanol flavonoids, like flavonols, while flavanols are present in lower quantities [10,17,33,34].

Local irradiance produced significant differences in antioxidant capacities and accordingly in amount of flavonoids and flavanols of sun acclimated and shaded leaves, except in the case of TEAC, TRC and flavanol content of G. White. Comparing the effect of sunlight on leaf flavonoid metabolites, G. Red accumulated 2.4-fold these compounds in sun leaves, followed by G. Altering (1.6-fold) and G. White (1.2-fold). Acclimation mechanism to sunlight resulted significantly higher values in G. Altering leaves compared to G. White and G. Red. Number of papers proved the effect of sun-, visible-, or UV-light on accumulation of polyphenolics [19,35], but their differences among grapevine varieties in their natural environment have not been compared yet.

Generally, plants exposed to higher solar radiation activate the synthesis of these secondary metabolites via the phenylpropanoid pathway controlled by transcription factors [36,37]. Despite of the same climatic conditions, agricultural practices and the close genetic background of studied varieties, they showed quantitative differences in accumulation of their flavonoid content. In consideration of these, the study of the transcriptional control of the process would explain the background of the different biochemical acclimations of the varieties.

Comparing antioxidant capacities and phenolic composition of leaves, berry skins and seeds

Antioxidant activities, flavonoid and flavanol contents of berry skins and seeds are shown in **Table 2**. Significant differences among berry skins of the varieties were detected in the case of FRAP and flavonoid content. The measured parameters were the highest in G. Red, followed by G. Altering and G. White. Regarding seeds, G. Red gave significantly lower FRAP and had lower flavonoid and flavanol content than the other two varieties, while TEAC and TRC were similarly high in all of the varieties.

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Table 2 Antioxidant Activities, Total Reducing Capacity, Flavonoid and Flavanol Content of Skins and Seeds of Gohér Varieties (Mean ± Standard Deviation). Minuscules Represent Groupings For Each Measured Parameter, Based On Dunnett Post-Hoc Tests.

Tissue	Variety	TEAC (μM TE/ 100 mg dw)	FRAP (μM ASE/ 100 mg dw)	TRC (mg GAE/g dw)	Flavonoids (mg QE/g dw)	Flavanols (mg CAT/g dw)
Skins						
	G. White	21.0 ± 0.96^a	74.7 ± 1.23^a	22.2 ± 0.83^a	44.4 ± 0.80^a	4.2 ± 0.25^a
	G. Altering	29.5 ± 0.73^a	104.7 ± 6.26^b	37.5 ± 1.07^a	63.8 ± 1.11^b	4.9 ± 0.41^a
	G. Red	24.1 ± 2.38^a	122.2 ± 3.91^c	46.3 ± 1.12^a	71.1 ± 2.11^b	4.9 ± 0.34^a
Seeds						
	G. White	390.5±11.92^a	49.9 ± 2.00^a	379.7 ± 21.72^a	101.7±2.65^a	65.6 ± 2.36^a
	G. Altering	405.7±16.22^a	51.3 ± 088^a	385.4 ± 45.89^a	113.9±4.64^a	69.9 ± 7.38^a
	G. Red	389.4 ± 2.53^a	37.9 ± 0.51^b	353.7 ± 17.74^a	93.4 ± 8.94^b	60.6 ± 3.87^b
In the same column mean values with different letters differ significantly ($p<0.05$)						

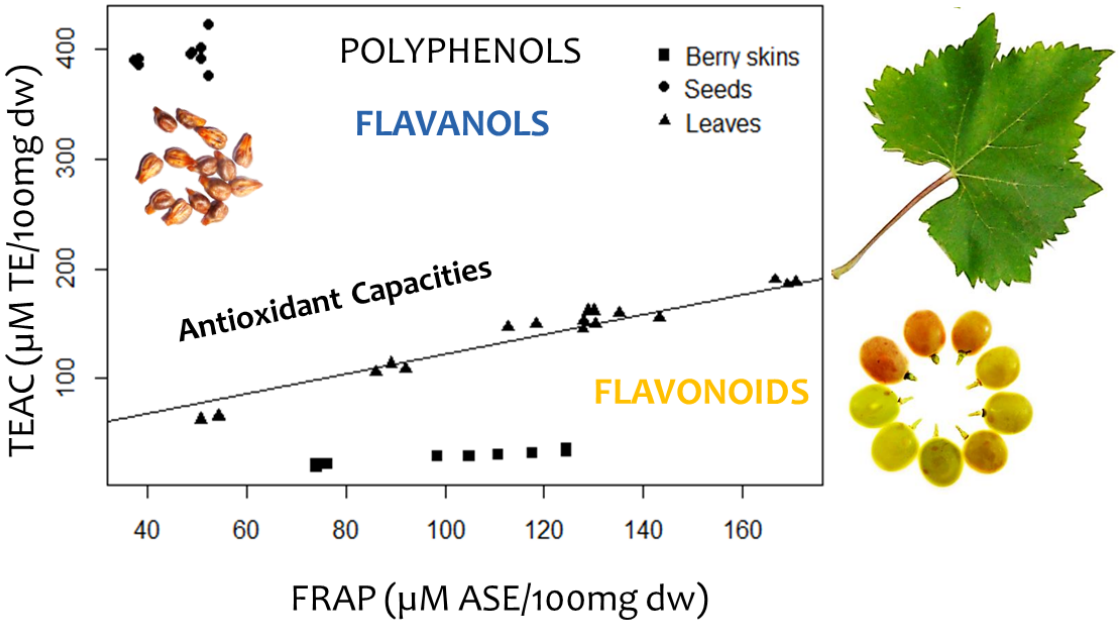

Figure 1 TEAC and FRAP of Different Grapevine Tissues Are Compared Pair Wise. Various Symbols Represent Various Tissue Types (Dots – Seeds, Triangles – Leaves, Squares – Berry Skins). The Trendline Represents The Tendency In The Measured Plant Part.

Seeds presented the highest TEAC and TRC among tissue types, while sun leaves showed the highest FRAP values **(Figure 1)**. TEAC and TRC of seeds were in average 1.7-2.4-fold and 12-15-fold higher than those of leaves and skins, respectively. Other studies have also reported the significantly higher TRC (named as Total Phenolics in the cited articles) of leaves and seeds compared to skins [13,32,38]. In contrast, Farhadi et al. (2016) [12] measured much higher total phenol content in berry skins than in leaves and seeds. Regarding FRAP values, sun leaves showed the highest potentials, which were in average 1.5-fold and 3-fold higher than those of skins and seeds, respectively. In agreement with our findings, Doshi et al. (2006) [39] reported significantly higher FRAP values in leaves and in other grapevine parts, than in berry skins.

Seeds showed the highest flavonoid content due to high amount of flavanols in this tissue compared to leaves and skins. Our results are in accordance to those obtained in other grapevine varieties [38,40]. Flavonoids were present in similar amounts in berry skins and leaves. Higher flavonoid content was detected in red grape berry skins in Farhadi et al. (2016) [12], presumably due to the high anthocyanin content of the skins, while the leaf samples contained less flavonoids compared to our results. Leaves contained about 5-fold higher amount of flavanons than skins, similarly to other *V. vinifera* varieties [32], while Taware et al. (2010) [17] detected significantly higher amount of catechin and epicatechin in skins than in leaves of the grapevine studied.

The high TEAC and TRC, but low FRAP of seeds as well as its vice versa of skins may be due to the different polyphenolic composition of the tissues studied. Proportion of flavanols in the flavonoid group showed remarkable differences. Flavanols in leaves, skins and seeds accounted respectively for 20-28%, 7-10% and 61-65% of flavonoids in the varieties. High concentration of flavanols, such as catechin and epicatechin in the seed samples, and high amount of other flavonoids, such as flavonols and anthocyanins in leaves and skins, proved by other studies [7,13,41].

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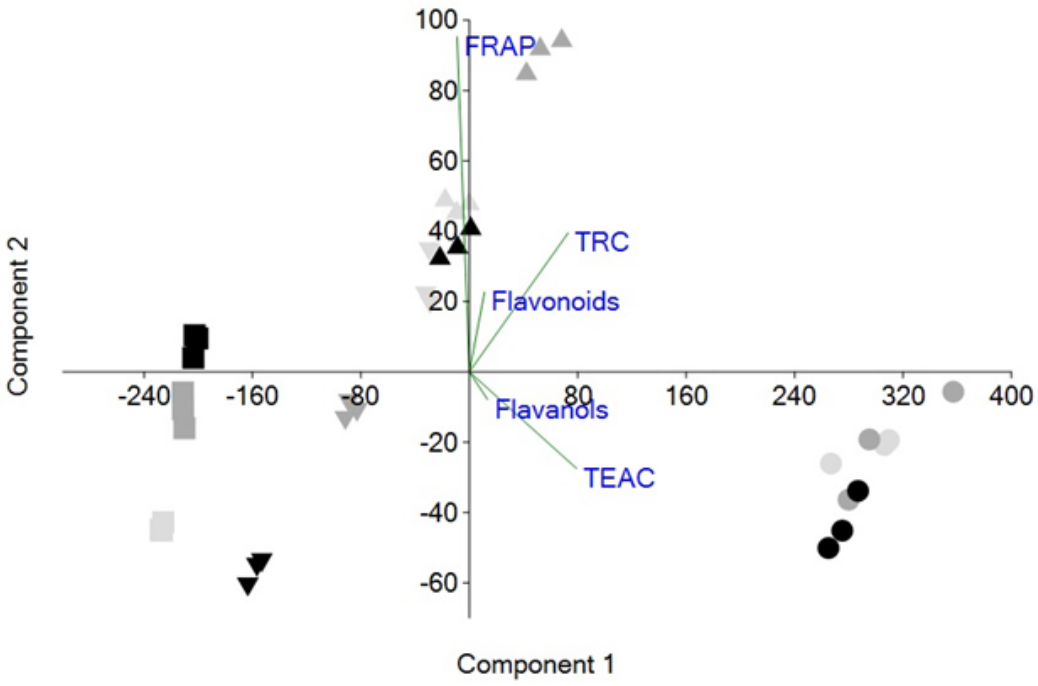

Figure 2 Principal Component Analysis of All Individuals Based On The Photometric Results. Distances Were Calculated Using Euclidean Distance Index. Various Symbols Represent Various Tissue Types (Dots – Seeds, Triangles – Leaves, Squares – Berry Skins), Colors Represent The Varieties (G. White – Grey, G. Altering – Dark Grey, G. Red – Black). Component 1 Explains The 94.80% Of Variance, While Component 2 Explains The 4.63% Variance Of The Data.

In our study, strong correlation was found between TEAC and TRC assays (R^2=0.9509, p<0.001) in accordance with the results of different plant extracts [42], while TEAC and FRAP showed no correlation (R^2=0.2075; p<0.01). Furthermore correlations between antioxidant capacities and groups of polyphenolics in different grapevine tissues were established **(Figure 2)**. According to the literature, phenolic profiling of grapevine leaves and skins showed high amounts of quercetin derivatives [13,43,44]. The high flavonol content (mainly quercetin derivatives) of berry skins and the high flavanol content of seeds had a significant influence on FRAP and TEAC, respectively. Leaves represented higher TEAC and higher FRAP values due to higher flavanol and flavonol content, than skins and seeds, respectively. Strong correlation of antioxidant activity with phenolic compounds was found in grapevine leaf extracts [15], in mushrooms [45] or in olives [46], nevertheless any correlation was observed in the case of Portuguese grapevine leaves [13]. Our results support the observation on correlation between antioxidant capacities and polyphenolic test compounds by Csepregi et al. (2016) [21], in so far as the antioxidant properties of phenolic rich samples depend on the assay used.

CONCLUSION

Our work reports the favorable antioxidant properties of grapevine leaves compared to berry skins and seeds, due to higher quantity of polyphenolic compound groups and varied chemical composition, respectively. The influence of variety and mainly the sunlight acclimation on the measured parameters of the leaves was emphasized. Furthermore, different sensitivity of antioxidant potentials to phenolic compound groups linked to different tissue types was established. Although the berry color variants are genetically in close relationship, variety and sun exposure both influenced the bioactivity and chemical content of the leaves in different manner. Skins differed significantly in their FRAP values and in non-flavanol flavonoid content, while seeds were less influenced by genetic factors. These results indicate that leaves are rich source of natural antioxidants with important economic impact in the industry.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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