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ORIGINAL PAPER

# Ripening of *Pithecellobium dulce* (Roxb.) Benth. [Guamúchil] Fruit: Physicochemical, Chemical and Antioxidant Changes

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**Abstract** The fruit of Guamúchil is an excellent source of bioactive compounds for human health although their natural occurrence could be affected by the ripening process. The aim was to evaluate some physicochemical, chemical and antioxidant changes in guamúchil fruit during six ripening stages (I to VI). A defined trend ($p \leq 0.003$) was observed for color [°Hue, 109 (light green) to 20 (dark red)], anthocyanins (+571 %), soluble solids (+0.33 °Brix), ash (+16 %), sucrose (−91 %), proanthocyanidins (63 %), ascorbic acid (−52 %) and hydrolysable PC (−21 %). Carotenoids were not detected and chlorogenic acid was the most abundant phenolic compound. Maximal availability of these bioactives per ripening stage ($p \leq 0.03$) was as follows: I (protein/ lipids/ sucrose/ proanthocyanidins/ hydrolysable phenolics), II (total sugars/ ascorbic acid), III (total phenolics), IV (flavonoids/ chlorogenic acid) and VI (fructose/ glucose/ anthocyanins). Color change was explained by sucrose ($\beta = 0.47$) and anthocyanin ($\beta = 0.20$) contents ($p < 0.001$). Radical scavenging capacity (ORAC, DPPH and TEAC) strongly correlated with total PC ($r = 0.49$–$0.65$, $p \leq 0.001$) but 89 % of ORAC's associated variance was explained by anthocyanin + sucrose + ascorbic acid ($p \leq 0.0001$). Guamúchil fruit could be a more convenient source of specific bioactive compounds if harvested at different ripening stages.

**Keywords** *Pithecellobium dulce* · Guamúchil · Ripening · Radical scavenging capacity · Phenolic compounds · Anthocyanins

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**Abbreviations**

| | |
|---|---|
| AAPH | 2,2'-azobis-2-amidinopropane |
| $ABTS^{\cdot-}$ | 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonate |
| DPPH | 2-diphenyl-2-picrylhydrazyl hydrate |
| EN | Essential nutrient |
| ORAC | Oxygen radical absorbance capacity |
| PC | Phenolic compounds |
| *PDf* | *Pithecellobium dulce* fruit |
| RSC | Radical scavenging capacity |
| Trolox | 6-hydroxy.2.5.7.8-tetramethyl-s-dicarboxylic acid |
| TE | Trolox equivalents |

## Introduction

*Pithecellobium dulce* (Roxb.) Benth is a semi-evergreen, small to medium size tree with a broad crown that belongs to the *Leguminosae-Mimosaceae* family. It is widely

distributed in tropical and sub-tropical regions of India, Southeast Asia and Latin America where is known as *Manilla tamarind, Madras thorn, Sweet tamarind, Makamted, Konapuli, Opiuma*, *Monkeypod, Khoya babla, Jilapi fol gach, Jungli jalebi, Kamachile, Guasima, Guamá americano*, *Pinzan* or *Guamúchil* [1–4]. One tree produces ~40 kg of dehiscent pods (65–75 % fruit, 9–13 % seed, 17–19 % peel) during a very short fruiting season and one pod contains 5–12 black seeds surrounded by a spongy edible fruit (*PDf*) called aryl. Mexicans, Thais and Indians consume white, pink or red *PDf*, either raw or in traditional drinks [1, 3, 4].

*PDf* is a good source of vitamins, dietary fiber and high quality protein. One hundred grams (86–93 kcal) of fresh ripe *PDf* is enough to fulfill between 100 and 320 %, 23–44 % and 18–32 % of the recommended dietary allowance of ascorbic acid, thiamine and dietary fiber for Mexicans 4 to 50 years old [1, 3, 4]. Semi-dried (15–18 % moisture) *PDf* contains 12–15 % protein with a higher content of lysine (7.8 %) and sulfur amino acids (2.8 %) than *Glycine max* or *Phaseolus vulgaris* [4–7]. From a pharmacognostic standpoint, all plant parts have been used to prepare several folk remedies for centuries [2]. Its phytochemicals exhibit several bioactivities with anti-inflammatory, anti-bacterial, anti-convulsant and anti-ulcer effects [2, 8]; most of these biological actions involve *PDf* free radical scavenging capacity (RSC) and other antioxidant mechanisms [9–14]. However, Asian [1, 5] and Mexican [3, 4] ripe *PDf* seem to differ in its composition of essential nutrients (EN) and phenolic compounds (PC). Nevertheless, to our knowledge, there are no reports on EN/PC changes in *PDf* during ripening. The aim was to evaluate some physicochemical, chemical and antioxidant changes of *PDf* during six ripening stages and to explore, by means of linear regression analysis, the dynamic changes in color and RSC of this underutilized legume from Northern Mexico.

## Material and Methods

**Reagents** Pure (≥93 %) standards [*D*-[+] isomers of glucose, fructose and sucrose, (+)-α, β-carotenes, gallic, chlorogenic, and caffeic acid, (+)-catechin, rutin, cyanidin chloride, and Trolox], radicals (DPPH, $ABTS^{\bullet-}$, AAPH), Folin–Ciocalteau phenol reagent, fluorescein, and ACS-grade salts and acids ($KH_2PO_4$, $NaNO_2$, $Na_2CO_3$, $FeCl_3.6H_2O$, $AlCl_3$, NaOH, KCl, HCl, $H_2SO_4$, $K_2S_2O_8$, $C_2H_3O_2Na$) were obtained from Sigma-Aldrich-Fluka (St. Louis, MO, USA). HPLC- or analytical-grade solvents (acetonitrile, tetrahydrofuran, methanol, ethanol, acetone and butanol) were obtained from JT-Baker (Avantor Performance Materials S.A. de C.V., Mexico).

**Plant Material** *P. dulce* pods were collected in four occasions (May to June) in a location nearby Hermosillo, Sonora, Mexico (29° 10′ 56″ *N*-110° 52′ 54″ *W*, 250 m ASL) and transported under refrigeration (4 °C) immediately to the laboratory. Pods were examined for integrity, insect contamination and absence of dust. Clean *PDf*s were carefully separated from their seeds and subjectively classified into six ripening stages according to their size and color (ESM 1): small/light green (I), medium/white (II), medium/pink (III), medium/50 % red (IV), medium/70 % red (V) and, medium/red ≥90 % (VI). 500 g of *PDf* per ripening stage were divided in two subsamples for further analyses in fresh- or dry-matter: 100 g fresh *PDf* (*PDf-FM*) were used to estimate all physicochemical parameters and 400 g were frozen (−80 °C) and freeze-dried (−42 °C, 114 militorr, 48 h; 12 XL Virtis-Sentry Freezemobile Freeze Dryer, Virtis Co., Inc. Gardiner, NY) to a constant weight in 1200 $cm^3$ flasks protected from light. All six freeze-dried samples (*PDf-DM)* were grinded to a fine powder (≤0.40 μm) and kept at -20 °C until use.

***PDf*-DM Extracts** Methanol (5 mL), hydro-methanol (20:80; 20 mL), acidified methanol (20 mL), ethanol (10 mL), tetrahydrofuran (25 mL) or $HPO_3$:$CH_3COOH$:water (30:80:90, *w*/*v*/v, 20 mL) extracts, were obtained at room temperature from 0.5–1 g of each *PDf-DM* samples, following Palafox-Carlos et al. [15] extraction procedures. Air-dried precipitates obtained from hydro-methanol extractions were further hydrolysed with 10 mL of $FeCl_3$: HCl 37 %: butanol (0.07:2.5:97.5 w/*v*/v) for 3 h at 100 °C to estimate the content of proanthocyanidins or with MetOH: $H_2SO_4$ (20:2, v/v) for 20 h at 85 °C to estimate hydrolysable PC content, under dark conditions and cooled at room temperature. All samples were either centrifuged (3000 rpm, 15 min, 2-5 °C) or directly filtered (0.22–0.45 μM) prior spectrophotometric or HPLC-analyses. Six replicates per sample (I-VI) were obtained.

**Physicochemical Analysis** 50 arils (*PDf-FM*) per stage (I to VI) were used to evaluate their weight (to the nearest ±0.001 g) and color using a colorimeter (CR-300, Konica Minolta Sensing, Inc., USA) and a Hunter Lab scale. Average *L**, *a** and *b** values were used to calculate [16, 17] hue angle [$^{o}Hue = Tang^{-1}$ ($b^*/a^*$)] and total color change as compared to stage I with the following formula: $\Delta E^* = [(L_o^*-L^*)^2 + (a_o^*-a^*)^2 + (b_o^*-b^*)^2]^{1/2}$. Also, 0.5 g of each *PDf-DM* samples were homogenized in distilled water (50 mL) and used to estimate total soluble solids ($^{o}$Brix), pH and titratable acidity (g citric acid / 100 mL).

**Proximate Analysis** Proximate analysis in triplicates (CV ≤ 10 %) was performed in *PDf*-DM samples, using official AOAC methods for ash, protein, fat, moisture and carbohydrate (by difference) content.

**HPLC Analysis** Ethanol soluble sugars (glucose, fructose and sucrose, g/100g DM) were analyzed by normal phase-HPLC [isocratic: acetonitrile: water (80:20, *w/v*)]/ refractive index detection according to Gonzalez-Aguilar et al. [18]. Total carotenoids (α + β, μg/100 g DM) extracted with tetrahydrofuran were analyzed by reverse phase-HPLC [isocratic = acetonitrile: methanol: tetrahydrofuran (58:35:7)]/ UV-VIS detection (460 nm) according to Mejia et al. [19]. Ascorbic acid (mg/100g DM) extracted with $HPO_3$: glacial acetic acid: water was analyzed by normal phase-HPLC [isocratic: $KH_2PO_4$: acetonitrile (25:75, *w/v*)]/ UV-VIS detection (268 nm) according to Donner and Hicks [20]. Methanol extractable-PC were analyzed by reverse phase-HPLC [gradient: acetonitrile (100 %): diluted acetic acid (1 %)]/ diode array detection (280 nm) according to Feregrino-Pérez et al. [21] with minor modifications in the gradient protocol and using gallic, caffeic and chlorogenic acids, (+)-catechin, rutin and quercetin as external standards.

**Phenolic Compounds (PC)** Total PC and flavonoids were extracted from *PDf*-DM samples with hydro-methanol (20:80) and analyzed in a FLUOstar Omega multifunctional microplate reader (BMG LABTECH, Inc. Durham, NC, USA) in UV-VIS spectrometer operation mode. PC content was assayed according to Singleton and Rossi [22] with the Folin-Ciocalteu reagent and expressed as mg of gallic acid equivalents per gram of DM (mgGAE/g DM) and total flavonoids according to Kim et al. [23] and expressed as mg of quercetin equivalents per gram of dry matter (mgQE/g). Precipitates from hydro-methanol extraction were used to evaluate the content of proanthocyanidins according to Reed et al. [24] and expressed as mg of cyanidin equivalents [25] per gram (mgCyE/g) and hhydrolysable PC according to Hartzfeld et al. [26], treated as for total PC, and expressed as mgGAE/g. Monomeric anthocyanins extracted with acidified methanol were evaluated by the AOAC (2005.02)-pH differential method [27] and expressed as milligrams cyanidin-3-gycoside (Cy3G) equivalents (mg Cy3GE/ g).

**Free Radical Scavenging Capacity (RSC)** DPPH (at 518 nm) and TEAC (at 734 nm) RSC assays were performed according to Brand-Williams et al. [28] and Re et al. [29] respectively, with minor modifications in reaction volumes as suggested by Palafox-Carlos et al. [15]. ORAC assay was performed according to Ou et al. [30] using fluorescein [10 nM, excitation (485 nm)/emission (520 nm)], AAPH (240 mM) and Trolox (0.006–0.2 μmol/mL, $R^2 \geq 0.95$) as standard. All values were expressed as mg (DPPH, TEAC) or μmol (ORAC) of trolox equivalents (TE)/g *PDf*-DM.

**Statistics** All variables were expressed as mean ± standard deviation (SD). One-way ANOVA followed by Tukey's *post-hoc* test was performed after testing data for skewness and kurtosis. Correlations were evaluated by Pearson's product-moment correlation (*r*) and simple and multivariate linear regressions were used to explain changes in color ($°Hue_{Ln}$) and RSC during *PDf* ripening, using response variables with a known role in color (*L*, a*, b**, anthocyanins, ascorbic acid, sucrose and ethanol-soluble sugars) or RSC (PC, ascorbic acid). Statistical differences were considered at $p < 0.05$. Statistical analysis was performed using NCSS 2000 (NCSS Statistical Software, Kaysville, UT, USA).

## Results and Discussion

Nowadays, nutrition-conscious consumers are seeking for organic and non-conventional plant foods in order to improve their daily intake of both EN and health-promoting phytochemicals, including PC. In order to fill this market gap, the food industry continuously searches for natural ingredients with a convenient combination of EN/PC within several ethnic crops. In this sense, *PDf* could be a very convenient source of EN/PC if it is harvested at different ripening stages as it will be explained in the following paragraphs.

The assessment of physicochemical changes during ripening is extremely important to decide when a given fruit should be harvested to ensure an acceptable sensorial quality [15]. Fruit's texture, flavor and color have a remarkable influence on consumer's acceptance. Mexican ripe *PDf* should have a smooth and fresh texture, sweet flavor and a pinkish-to-red hue [3, 4], associated with several physicochemical and functional changes during ripening. In this study, except for moisture (~72.5 %, $p = 0.12$), blueing ($b^*$) and weight loss (−45 %, only noticeable from stage V to VI), all other physicochemical parameters showed significant changes ($p_{trend} \leq 0.001$) during ripening (ESM 2a,b): pH (−0.3 units), total soluble solids (+0.33 °Brix) and color (°Hue, 109 to 20; $\Delta E^* = 71.6$) from a lighter-green ($L_o^* = 83.9$, $a_o^* = -10.2$) to a darker-red ($L^* = 31.2$, $a^* = 33.7$). Mexican white and red [3] or Indian white and pink [5] ripe *PDf* have almost the same physicochemical characteristics as *PDf* at stages III to V reported here. However, average titratable acidity was lower than that reported by Pio-León et al. [3] in *PDf* samples collected 580 km south (24° 46′ 02″ *N*-107° 41′ 40″ *W*) at a higher altitude (450 m ASL). Nevertheless, even when the time of harvest is different due to differences in altitude, these results indicate that both crops are quite similar.

From a nutritional standpoint, the apparent energy content of *PDf*-DM did not change during ripening (394 ± 1 kcal/ 100 g). However, except for total carbohydrate (81–83 % as dry matter, $p = 0.17$), all other chemical parameters changed during ripening ($p \leq 0.03$; ESM 3): protein (−11 %), lipids (−42 %), sucrose (−92 %) and ashes (+16 %). The latter could be explained by a significant increment in certain minerals

**Table 1** Simple linear regression analysis

| | | m | b | $R^2$ | *p* |
|---|---|---|---|---|---|
| °Hue $_{Ln}$ | *a** | -1.5 | 87.0 | 0.88 | ≤0.0001 |
| | Anthocyanins [a] | -1.1 | 4.5 | 0.67 | ≤0.0001 |
| DPPH | Total PC | 4.5 | -14.9 | 0.65 | ≤0.0001 |
| | Anthocyanins | -4.0 | 18.1 | 0.24 | 0.050 |
| TEAC | Total PC | 2.8 | -4.4 | 0.49 | 0.0001 |
| ORAC | Total PC | 11.8 | 66.5 | 0.49 | 0.002 |
| | Ascorbic acid | 0.05 | 123.5 | 0.49 | 0.050 |
| | Anthocyanins | -14.9 | 155.3 | 0.28 | 0.024 |

[a] Phenolic compounds (PC)

such as calcium, iron, potassium, sodium and zinc, as reported by Pio-León et al. [3] in reddish *PDf*. Most changes occurred between the first two stages, just as the pod opens (shatering) and its photosynthetic capacity decreases, as judged by *PDf*'s green color disappearance (ESM 1). *PDf*'s protein content at any stage seems to be higher than previously reported [4] but lower than that of soybean [6] or dry beans [7] or *Pitecellobium jiringa* [31]. Also, despite the fact that *PDf* loses 252 mg of ascorbic acid during ripening (−52 %), the average content (mg/100 g) in *PDf*-FM (calculated from *PDf*-DM values) is many times higher than that of apples, plums, mangoes, oranges and litchis and active vitamin C (ascorbic acid + dehydroascorbic acid) could be higher than previously reported [1, 3]. Gathering all these findings plus the fact that non-ethanol-soluble sugars represent more than 50 % of all carbohydrates (most of them dietary fiber) at any ripening stage [4], the low calorie/nutrient dense nature of *PDf*-DM makes it an ideal ready-to-eat snack or a food ingredient (if grinded) for individuals with insulin-resistance or dyslipidemias [32], particularly in its first two ripening stages.

The reduction in ascorbic acid and sucrose contents with an apparently steady-state in glucose and fructose levels is noteworthy (ESM 3). It seems likely that the observed peak level of ascorbic acid, from stage I to II and the steady state level in sucrose at the same time, could be the result of a lesser pod's photosynthetic capacity and the exposure of *PDf* to environmental stressors since ascorbic acid increases for plant defense in absence of more complex antioxidant molecules produced during phase II metabolism. From stage III-VI, the channeling of fructose and glucose (from sucrose breakdown) to produce cell wall polymers to support *PDf* growth (explained by a higher size, ESM 1) in semi-open pods plus the production of specialized metabolites during phase II metabolism (*e.g.*, anthocyanins), seems to reduce the ascorbic acid and sucrose pools. Lastly, the total carotenoid content was below detection level (≤0.1 μg/g) at any stage, as commonly found in raw legumes.

PC have been considered the most important and ubiquitous molecules in the plant kingdom, responsible for the RSC of many edible plants. While many EN are produced, accumulated and metabolized during phase I plant metabolism, PC are produced during phase II. In this study, changes in total PC and subgroups (total flavonoids, proanthocyanidins, hydrolysable PC, and anthocyanins) were statistically different between stages ($p \leq 0.003$). However, a positive or negative trend associated with ripening for total PC and total flavonoids was not as evident (ESM 4) as for anthocyanins (0.21 to 1.41 mg Cy3GE, +571 %), proanthocyanidins (1.66 to 0.71 mgCyE, −63 %) or hydrolysable PC (0.58 to 0.46 mgGAE, −21 %). Pearson's product-moment correlation (*r*) evidenced a strong relationship ($p \leq 0.001$) between $L^*$ and °Hue with anthocyanins ($r = -0.92$ and $-0.71$), ascorbic acid ($r = 0.91$ and 0.71) or sucrose ($r = 0.99$ and 0.89) while the opposite was observed for *a** ($r = 0.65$, $-0.66$, $-0.88$, $p \leq 0.01$). $L^*$ was mildly associated with ethanol-soluble sugars changes ($r = 0.57$) and blueing (*b**) with anthocyanins ($r = -0.60$) or °Hue ($r = 0.50$). The logarithmic (Ln) transformation of °Hue values (°Hue $_{Ln}$) to improve its normality showed that 88 % ($p \leq 0.001$) of its associated variance was explained by *a** (Table 1) and an additional 9 % by *b** (*a** + *b**, Table 2) but $L^*$, anthocyanins and ascorbic acid lost statistical significance in this model. However, anthocyanins alone (Table 1) or combined with sucrose (Table 2) explained 67 to 89 % of

**Table 2** Multivariate linear regressions

| Model | Intercept | β1 | β2 | β3 | $R^2$ | *p* |
|---|---|---|---|---|---|---|
| °Hue $_{Ln}$ | | | | | | |
| *a** + *b** | 50.9 | -1.4 | 1.5 | | 0.97 | ≤0.0001 |
| Anthocyanins + Ascorbic acid | 3.0 | -0.5 | 0.003 | | 0.70 | ≤0.0001 |
| Anthocyanins + Sucrose | 2.7 | 0.2 | 0.5 | | 0.89 | ≤0.0001 |
| ORAC | | | | | | |
| Total PC [a] + Ascorbic acid | 66.3 | 10.2 | 0.03 | | 0.54 | 0.004 |
| Total PC + Anthocyanins | 87.8 | 9.3 | -7.2 | | 0.53 | 0.005 |
| Total PC + Ascorbic acid + Anthocyanins | 68.6 | 10.0 | 0.03 | -0.78 | 0.54 | 0.015 |
| Anthocyanins + Sucrose + Ascorbic acid | 2.5 | 0.3 | 0.5 | 0.0005 | 0.89 | ≤0.0001 |

[a] Phenolic compounds (PC)

°$Hue_{Ln}$ associated variance ($p < 0.001$). Anthocyanins are probably the most important flavonoid subgroup in ripe *PDf*, although they have been scarcely reported in scientific literature and our results are consistent with these studies [3, 5]. Nevertheless, anthocyanins changes during the ripening of *PDf* are reported here for the first time.

The apparent steady state in total PC and total flavonoids may resulted from *de novo* biosynthesis and/or from polymeric PC breakdown, as judged by the significant increments in chlorogenic acid, catechin (possibly from breakdown of proanthocyanidins) and gallic acid (from breakdown of hydrolysable PC) contents from stage II to V (ESM 4). Regardless of intrinsic methodological differences, many authors have reported gallic, mandelic, ellagic, ferulic and *p*-coumaric acids and naringin, quercetin, kaempferol, daidzein and rutin but no chlorogenic acid or catechin [11, 13]. Moreover, the ratio of phenolic acids (~60 mg/g DM, mainly gallic, caffeic and *p*-coumaric acids) and flavonoids (~203 mg/g DM, mainly luteolin and myricitin) present in Thai ripe *PDf* [1] seems to be inverted in the ripe (V-VI) *PDf* studied here. Many factors are involved in these differences including, but not restricted to, environmental and agronomical factors and plant's genomic programming that can modify *PDf* nutraceutical potential. This fact deserves a more detailed comparison in a future study. For now, Mexican *PDf* is an excellent source of chlorogenic acid, a phenolic acid with anti-diabetic, anti-lipidemic and fatty liver attenuating properties [32, 33].

Lastly, RSC is the result of a concerted action of all antioxidant species present in a sample. However, the selection of a single method to evaluate RSC would not accurately reflect the complexity of all chemical interactions *in vivo* and thus more than one method should be selected [15]. Three RSC methods were selected here: DPPH (stable radical), TEAC (radical cation) and ORAC (peroxyl radicals) which have been extensively used in plant biochemistry and to evaluate other biological specimens. In this study, two interesting findings emerged from RSC assays: a) *PDf* at stage III (ESM 5) showed the highest RSC [22.3 (DPPH) and 19.9 (TEAC) mg TE and 159.7 (ORAC) μmol TE/g DM] and b) ORAC's associated variance was explained by total PC or ascorbic acid alone (49 %, $p \leq 0.05$; Table 1) or both (54 %, $p = 0.04$; Table 2) by anthocyanins + sucrose + ascorbic acid (89 %, $p \leq 0.0001$; Table 2). These results could be explained by the same argument: PC with mild (ascorbic acid, proanthocyanidins and hydrolysable PC) and high (anthocyanins, chlorogenic and gallic acid) RSC counteract at stage III (ESM 4) resulting in the highest value. In agreement with our results, Pio-León et al. [3] reported values of DPPH and TEAC (as mg equivalents of ascorbic acid /100 g PF) much higher in red (223 and 225) than white (171and 156) *PDf*. It is well known that RSC of a complex mixture of PC can be enhanced or reduced by several synergistic or antagonistic actions between molecular species and their structural elements, particularly the number and position of hydroxyl groups [34]. However, the extent to which this phenomenon occurs during PDf ripening deserves a future study.

## Conclusion

Maximal availability of essential nutrients (EN) and phenolic compounds (PC) in *PDf* is affected by the ripening process ($p \leq 0.03$), being more nutrient-dense at earlier stages (I-II) but richer in monomeric PC afterwards (III-VI) and so, *PDf* could be a more convenient source of these bioactives if it is harvested at different ripening stages. This evidence could be translated into a more profitable agronomic exploitation of this ethnic fruit, by using more standardized and sophisticated horticultural procedures instead of its manual collection with hooks and bags. However, further studies are needed to evaluate the biological impact (either pro- or anti-physiological) of all chemical, physicochemical and antioxidant changes in order to formulate novel nutraceuticals based on *PDf* bioactives.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interest

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