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Flavonoids and phenolic acids from pearl millet (*Pennisetum glaucum*) based foods and their functional implications

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ABSTRACT

Background: Pearl millet (*Pennisetum glaucum*), considered a poor man's cereal, may be a repository of dietary antioxidants, especially flavonoids and phenolic acids, which provide bioactive mechanisms to reduce free radical induced oxidative stress and probably play a role in the prevention of ageing and various diseases associated with oxidative stress, such as cancer, cardiovascular, and neurodegenerative diseases.

Objective: The present study focused on the identification of individual flavonoids and phenolic acids from seven commercial varieties of pearl millet and five samples of pearl millet-based traditional recipes of Banaskantha, Gujarat, India.

Methods: Total phenols were determined by the Folin-Ciocalteu method, and individual polyphenol separation included the isolation and identification of (a) flavonoids, (b) phenolic acids, and (c) glycoflavones involving interaction with diagnostic reagents and paper chromatographic separation of compounds and their UV-visible spectroscopic studies including hypsochromic and bathchromic shifts with reagents such as AlCl3, AlCl3/HCl, NaOMe, NaOAc, and NaOAc/H3PO3. Five traditional recipes consumed in the pearl millet producing belt of Banaskantha, Gujarat, India, were standardized in the laboratory and analyzed for phenol and individual flavonoids.

Results: Total phenols in raw samples ranged from 268.5 - 420mg/100g of DW and 247.5 - 335mg/100g of DW in cooked recipes. The commonly identified flavonoids were tricin,

acacetin, 3, 4 Di-OMe luteolin, and 4-OMe tricin. Five phenolic acids were identified: namely vanilic acid, syringic acid, melilotic acid, para-hydroxyl benzoic acid, and salicylic acid.

Conclusion: The presence of flavonoids, such as tricin, acacetin, 3, 4 Di-OMe luteolin, and 4-OMe tricin, indicate the chemopreventive efficacy of pearl millet. They may be inversely related to mortality from coronary heart disease and to the incidence of heart attacks in the pearl millet consuming belts of the world.

Keywords: Polyphenols, Antioxidant, Flavonoids, Total Phenols, Pearl Millet (raw and cooked) *(Pennisetum glaucum),* Banaskantha, Gujarat.

INTRODUCTION:

Pearl millet *(Pennisetum glaucum* (L.) R. Br.*),* is the most popular cereal crop grown in tropical semi-arid regions of the world. Locally known as *Bajra* in Gujarat, a western state of India, the total area in which pearl millet is produced in Gujarat is 7033 ha [2008-2009] [1]. Gujarat ranks third in pearl millet yield compared to all other states in India [2]. In Gujarat, the highest production is found in the northern plains of Banaskantha (2558 mt). Banaskantha is one of the 25 administrative districts, located in the northeast part of Gujarat. It produces 26.61% of pearl millet, followed by wheat (11.7%), as per the season crop report of 2007-2008 of Gujarat Government.

The commonly consumed recipes in Banaskantha are pearl millet based, namely; *rotla, khichadi, kuler, ghes,* and *matar,* since this cereal occupies major area and yield among different Kharif crops (planted for autumn harvest, may also be called the summer or monsoon crop in India, sown with the beginning of the first rains in July, during the south-west monsoon season) followed up by pulses such as Cicer arietinum L. (chickpea), Vigna radiata (L). Wilczek (green gram), Vigna mungo (L.) Hepper (black gram), Pisum sativum L. (field pea) and Cajanus cajan (L.) Millsp. (red gram or pegion pea) and *Sorghum bicolor* (sorghum, *jowar*). Among the 31 varieties of pearl millet recorded from this district, Dhanya 7882, Pioneer 86M52, Daha Grade seeds 555, Pioneer 86M64 (summer and Monsoon), and Pioneer 9444 are popularly consumed by the majority of people of Banaskantha district of Gujarat [*3*].

Though this cereal is rich in energy, protein, fat, and iron (NIN 2003) [4], no studies are available on the values of the total and individual polyphenol content of raw and cooked Indian pearl millet (commonly known as *bajra*) based recipes of Banaskantha, Gujarat.

Dietary antioxidants present in commonly consumed foods, especially the flavonoids and phenolic acids such as quercetin, kaempferol, and acacetin, and phenolic acids: vanillic acid, ferulic acid (cis and trans form), and p-coumaric acid, melilotic, and provide bioactive mechanisms to reduce free radical induced oxidative stress and their probable role in the prevention of ageing and various diseases associated with oxidative stress, such as cancer, cardiovascular and neurodegenerative diseases [5-9].

Pearl millet has been recommended for several therapeutic purposes, as it has been found to inhibit tumor development [10], control blood pressure and plasma low density lipo protein cholesterol levels [11], and possesses anti-allergenic characteristics. Due to its high fiber content,

pearl millet is also recommended for the treatment of severe constipation, stomach ulcers, and weight loss Its nutrient and non-nutrient (especially the phytochemicals) database, which may represent the underlying mechanism of these nutraceutical effects, needs to be established. The present study assessed the total phenol contend and identified individual polyphenols of raw and cooked pearl millet based recipes consumed in Banaskantha district of Gujarat, a western Indian state.

METHODS AND MATERIALS:

Sampling of raw pearl millet seeds. Based on the desk review and personal interviews with key informants and focus group discussions described in our earlier report [12], a total of 31 varieties of pearl millet were identified in Gujarat. Of these six popular varieties of pearl millet from private firms in Banaskantha were selected for the present study. These were Dhanya 7882, Pioneer 86M52, Daha grade seeds 555, Pioneer 86M64 summer, Pioneer 86M64 monsoon, and Pioneer 9444 (Table 1). Another sample of mixed variety and a commercially milled flour of mixed variety were also selected.

One kg of seeds for each variety and commercially milled flour were collected from the 30 villages of three blocks of Banaskantha district in Gujarat, packed in clean new polyethylene plastic bags, labeled, and brought to the laboratory. Same varieties were mixed and analyzed in the laboratory for total and individual phenols.

Table 1: Characteristics and Origin of the 13 Samples of Pearl Millet Selected for the Present Study

S. No.	Name of the variety	Type	Colour	Origin
1.	Dhanya, 7882	Raw, local variety	Irregular greyish seeds	Gujarat
2.	Pioneer, 86M52	Raw, local variety	Irregular shaped greyish seeds	Gujarat
3.	(DAHA) Grade Seeds 555	Raw, local variety	Light and dark green round granules	Gujarat
4.	Pioneer 86M64, Summer	Raw, local variety	Light green round pellets	Gujarat
5.	Pioneer 86M64, Monsoon	Raw, local variety	Irregular shaped greyish seeds	Gujarat
6.	Pioneer 9444, Summer	Raw, local variety	Light green and grey granules	Gujarat
7.	Mixed Variety (Dhanya 7882, Pioneer 86M52, (DAHA) Grade Seeds 555, Pioneer 86M64 (Summer), Pioneer 86M64 Monsoon, Pioneer 9444, Summer + LST GHB558, LST GHB 719, LST GHB 732, LST GHB 538, LST GHB 915, LST GHB 744)	Local and varieties developed by SDAU	Irregular green and grey granules	Gujarat

Sampling for cooked recipes. Based on the data collected from the ongoing project on the consumption patterns of pearl millet *(Pennisetum glaucum)* from 1077 women residing in 30 clusters of pearl millet belt of Banaskantha, Gujarat, India (using a qualitative research methodology-focus group discussion), the five most popularly consumed pearl millet based foods - *rotla, khichadi, kuler, ghes,* and *sukhadi* (*matar*) were selected for the estimation of total phenols and individual polyphenol identification (Table 2).

Five records for each recipe were noted from 6 villages selected on the basis of random sampling wherein the local women prepared the pearl millet based recipes in their traditional manner. These were standardized in the field by our field investigators with the help of standard cups (size C1 (200ml), C2 (100ml), C3 (80ml) and C4 (50 ml) and spoons (size 1Tbsp, 1Tsp, 1/2Tsp and ¼ Tsp) along with video recordings. These recipes were brought to the laboratory, dried in a hot air oven, and stored in autoseal polythene plastic pouches kept in airtight containers until further analysis was completed at room temperature.

Table 2: Characteristics and Origin of the Cooked and Processed Samples of Pearl Millet Selected for the Present Study

S.no	Name of the Sample	Sample preparation	Origin
1.	**Rotla**	Milled pearl millet with added salt, prepared as a dough, rolled, and roasted on dry heat; eaten regularly as a bread substitute	Collected from the field households
2.	**Matar**	Milled pearl millet, roasted with ghee and jiggery; a sweet dish prepared occasionally	Collected from the field households
3.	**Khichadi**	Soaked and cooked pearl millet in open/closed pan with addition of salt and spices; a regular meal	Collected from the field households
4.	**Kuler**	Milled pearl millet, mixed raw with ghee and jiggery; a sweet dish prepared occasionally	Collected from the field households
5.	**Ghes**	Hand pounded, cooked with water and buttermilk; a regular meal	Collected from the field households
6.	**Flour**	Commercially machine milled pearl millet	Collected from the field households

Chemicals. Sodium carbonate, copper sulphate – sodium potassium tartarate solution ($CuSO_4.5H_2O$), Folin-Ciocalteu reagent, dilute methanolic extract (0.5 ml of 1:10 g ml^{-1}), gallic acid (standard phenolic compound), (conc. HCL:acetic acid:water; 30:30:10) or glacial acetic acid, Na_2CO_3, $FeCl_3$, benzene, acetic acid, sodium formate, formic acid, para nitra aniline, and sulphanic acid were purchased from Durga Scientific Pvt. Ltd, Vadodara, Gujarat, India.

Solutions. Benzene:acetic acid:water (6:7:3, upper organic layer) in the first direction and sodium formate:formic acid:water (10:1:200) in the second direction were used as irrigating

solvent in phenolic acid estimation using paper chromatography. For diazotization in phenolic acid, 0.7g of p-nitraniline/sulphanilic acid were used.

Identification and quantification of individual phenols and total phenols. Identification of individual polyphenols was carried out as per *Mabry et al*, 1970 [13] using paper chromatography. The standard analytical procedures involving interaction with diagnostic reagents, i.e. spectroscopic grade methanol and paper chromatographic separation of compounds and their UV-visible spectroscopic studies including hypsochromic and bathochromic shifts with reagents such as $AlCl_3$, $AlCl_3$/HCl, NaOMe, NaOAc, and NaOAc/H_3PO_3, were followed for the identification of flavonoids and other phenolics. The UV absorption spectra of these compounds were recorded in methanol using 'Perkin – Eluer Lambda 25 UV/V_B spectrophotometer. The identities of all the compounds (flavonoids and phenolic acids) were confirmed by paper chromatography with authentic samples [13, 14, 15].

Determination of the total phenol content. Total phenols were determined by Folin-Ciocalteu method *Lowry et al, 1951* [16] and measured at 765 nm. The results are given in lg of Gallic acid equivalents (GAE) per g dry weight (DW) [17]. The Folin-Ciocalteu method for determination of phenolic compounds is similar to antioxidant activity determination; therefore, the values should at least partially express antioxidant activity [18].

RESULTS AND DISCUSSION:

Total phenol content in raw and cooked pearl millet. The total phenol content of raw varieties ranged from 268.5 to 420mg/100g, the highest in Pioneer 9444 and lowest in Dhanya 7882. Total phenols in cooked pearl millet based recipes ranged from 247.5-335mg/100g, the highest levels in *khichadi* and lowest in *matar*. A 9.33% difference was recorded indicating a reduction in total phenol in all cooked samples as compared to the raw varieties (Table 3 and 4).

Table 3: Results of Total Phenol from Raw Pearl Millet Samples

S. No.	Varieties of pearl millet	Total phenols (mg/100g DW)
1.	Dhanya, 7882	268.5
2.	Pioneer, 86M52	330
3.	(DAHA) Grade Seeds 555	332.5
4.	Pioneer 86M64, Summer	270
5.	Pioneer 86M64, Monsoon	315
6.	Pioneer 9444, Summer	420
7.	Mixed variety	330
8.	Flour (machine milled)	315
	Mean ± SD	**322.6**
	RANGE	**268.5 – 420**

On average, processed/cooked recipes of pearl millet in the present study had lower total phenol content then their unprocessed counterparts. Among the cooked samples, *khichadi*, which

is subjected to processing methods like soaking, had higher total phenol content compared to other cooked samples, which were subjected to milling and roasting. The results of total phenols (273mg/100g and 306.7 to 669.4mg/100g) are comparable with those of different investigators including *Singh et al and Samia et al* [19-20]. Other investigators [21-24] have stated that the different processing method significantly reduced the polyphenol content. Reduction in polyphenol may be attributed to the removal of the outer layer of the grain, which was reported to be rich in polyphenols, possibly attributable to the presence of phenolic oxidase during germination. The decrease in polyphenols during fermentation of processed grains indicates the ability of micro flora to ferment phenolics, as reported by *Bravo et al* [25].

Table 4: Results of Total Phenol for Cooked Pearl Millet Based Recipes from Banaskantha

S. No.	Names of the pearl millet based cooked recipes	Dry matter (%)	Total phenol (mg/100g)
1.	*Rotla* (milled and roasted on dry heat)	62	317.5
2.	*Matar* (milled, roasted with ghee and jaggery)	94	247.5
3.	*Khichadi* (soaked and cooked in open/closed pan)	38	335
4.	*Kuler* (milled and mixed raw with ghee and jaggery)	94	297.5
5.	*Ghes* (hand pounded, cooked with water and buttermilk)	34	265
	Mean	**64.4**	**292.5**
	Range	**38-94**	**247.5 – 335**

The polyphenols compounds are usually present in the grains of cereals [26-27]. They affect the (28) bioavailability of the minerals [29-32], the digestibility of carbohydrates [33-35], and inhibit the activity of proteolytic and amylolytic enzymes [36-39].

The method of extracting components from plant material is an important factor in measurements of individual phenols and total phenols. Therefore, before analyzing the individual and total phenols, the plant material was extracted with methanol. *Park and Chin* [40] recently observed that methanol extracted garlic had a greater TPC, DPPH radical scavenging activity, and reducing power than water extracted. *Strail et al* [41] used an aqueous methanol (1:1, v/v) to extract polyphenols from various plants, including Allium species.

Individual flavonoids identified in raw and cooked pearl millet. The flavonoids identified in raw and cooked pearl millet based traditional recipes (namely *rotla, khichadi, kuler, ghes,* and *matar)* were tricin, acacetin, 3-OMe tricin and 3, 4 – di-OMe luteolin (Table 5 and 6). Among the six varieties of grains, Tricin, Acacetin, and a new compound 3, 4 – di-OMe luteolin were identified in Pioneer 86M64, summer variety. In all the cooked samples, Tricin was commonly reported; however, acacetin and 4-OMe Tricin were absent in *kuler* and *rotla*. Flavonoids such as kaempferol, 4- OMe Kaempferol, 7'4 –di-OMe Kaempferol, Quercitin, 3'-OMe Quercitin, 4-

OMe Quercitin, 3,4-di-OMe Quercitin, gossypetin, quercetagetin, proanthocyanidin, anthocyanidin, and coumirins were not identified in raw or cooked pearl millet (Table 5).

Table 5: Results of Flavonoids from Raw Pearl Millet Samples

S. No.	Sample Name	Visible UV Color		λ max	Flavanoid Present
Private Varieties					
1.	Dhanya, 7882	Colourless	Brown	347.93	Tricin
2.	Pioneer, 86M52	Colourless	Brown	347.93, 326.96, and 269.95	Tricin, Acacetin
3.	(DAHA) Grade Seeds 555	Colourless	Brown	341.69, 268.50	Tricin, 4-Ome Tricin
4.	Pioneer 86M64, Summer	Colourless	Brown	342.65 and 268.32	4-OMe Tricin
5.	Pioneer 86M64, Monsoon	Colourless	Brown	343.86 and 268.58 341.06 and 269.32 329.85 and 269.75	Tricin, 3, 4- Di-OMe luteolin, Acacetin
6.	Pioneer 9444, Summer	Colourless	Brown	347.80 and 276.99 346.48 and 270.89 332.95 and 269.79	3, 4- Di-Ome luteolin, Acacetin
7.	Mixed Variety	Colourless	Brown	349.65 and 269.43 343.02 and 269.88 327.06 and 270.17	Tricin, Acacetin, 4-Ome Tricin
8.	Flour	Colourless	Brown	346.14 and 269.17 342.76 and 269.72	Tricin, 4-Ome Tricin

Table 6: Results of Flavonoids for Cooked Pearl Millet Based Recipes from Banaskantha

S. No.	Sample Name	Visible UV Color		λ max	Flavanoid Present
1.	Rotla (milled and roasted on dry heat)	Colourless	Brown	347.93	Tricin
2.	Matar (milled, roasted with ghee and jaggery)	Colourless	Brown	347.93 342.65 and 268.32 326.96 and 269.95	Tricin, Acacetin, 4-Ome Tricin
3.	Khichadi (soaked and cooked in open/closed pan)	Colourless	Brown	349.65 and 269.43 343.02 and 269.88 332.95 and 269.79	Tricin, Acacetin, 4-Ome Tricin
4.	Kuler (milled and mixed raw with ghee and jaggery)	Colourless	Brown	346.14 and 269.17 342.76 and 269.72	Tricin, 4-Ome Tricin
5.	Ghes (hand pounded, cooked with water and buttermilk)	Colourless	Brown	349.65 and 269.43 342.65 and 268.32 326.96 and 269.95	Tricin, Acacetin , 4-Ome Tricin

Tricin is abundant in millet, oats, and wheat. Tricin has been shown to have anticancer effects in the lower gut [42]. Moreover, Tricin has a relaxant effect on smooth muscle of intestinal tissues, powerful antioxidant effects [43], potent antihistaminic activity [44], and growth inhibition of human malignant breast tumor cells and colon cancer cells [45-46]. In addition, Tricin was recently shown to interfere with murine gastrointestinal carcinogenesis and is considered safe for clinical development as a cancer chemopreventive agent [47]. An acylated Tricin glycoside from sugarcane (*Saccharumofficinarum* L., Gramineae) juice exhibits *in vitro* antiproliferative activity against several human cancer cell lines with a higher selectivity toward cells of breast-resistant NIC/ADR line [48]. Flavonoids have been shown to modify eicosanoid biosynthesis (antiprostanoid and anti-inflammatory responses), protect low density lipoprotein (LDL) from oxidation (prevention of atherosclerotic plaque formation), and promote relaxation of cardiovascular smooth muscle (antihypertensive, antiarrhythmic effects). In addition, flavonoids have been shown to have antiviral and anticarcinogenic properties [49].

Flavonoids as antioxidants may inhibit the oxidation of LDL cholesterol, reduce platelet aggregation, or reduce ischemic damage. Since flavonoids have good antioxidant property, they are referred to as "nature's biological response modifiers", because they modify the body's reaction to pathogens as well as compounds such as allergens and carcinogens. They are powerful antioxidants giving protection against oxidative and free radical damage. They prevent formation of oxidized cholesterol through antioxidant effects. Flavonoids exert greater antioxidant effects than vitamin C, vitamin E, selenium, and zinc. Epidemiological studies have shown that flavonoid intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks, and that certain flavonoids can protect LDL from being oxidized and prevent atherosclerosis. In addition, a great number of plant medicines contain flavonoids, which have been reported by many authors as having antibacterial, anti-inflammatory, antiallergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic, and vasodilatory actions [50].

Table 7: Results of Phenolic Acid from Raw Pearl Millet Samples

S. No.	Sample Name	Phenolic Acid Present
1.	Dhanya, 7882	Syringic, salicylic, p-OH benzoic acid
2	Pioneer, 86M52	Syringic, salicylic, p-OH benzoic acid
3	(DAHA) Grade Seeds 555	Vanillic, syringic, p-OH benzoic acid
4.	Pioneer 86M64, Summer	Vanillic, p-OH benzoic acid
5.	Pioneer 86M64, Monsoon	Vanillic, syringic, melilotic acid
6.	Pioneer 9444, Summer	Vanillic, syringic, melilotic acid
7.	Mixed variety	Vanillic, syringic, salicylic, melilotic acid
8.	Flour	Vanillic acid, syringic acid, p-OH benzoic acid, o-coumaric acid, p-coumaric acid

Phenolic acids identified in raw and cooked pearl millet. Phenolic acids identified in raw samples (powdered in the laboratory using electric grinder) were vanillic, syringic, salicylic, p-OH benzoic acid, and melilotic acid, and in commercially milled flour sample were vanillic, syringic, p-OH benzoic acid, o-coumaric, and p-coumaric. Commercially milled pearl millet flour was identified as having a high quantity of phenolic acids when compared to raw samples. Phenolic acids identified in cooked recipes were vanillic, syringic, melilotic, Cis Ferulic, p-OH benzoic, and p-coumaric. Among all the cooked samples, the majority of phenolic acids were identified in *ghes*. Cis Ferulic was the unusual phenolic acid identified in *rotla* (Table 7, 8 and 9). There is no literature available regarding the phenolic acid in these cooked samples of pearl millet.

Table 8: Results of Phenolic Acids for Cooked Pearl Millet Based Recipes from Banaskantha

S. No.	Sample	Phenolic Acid Present
1.	Rotla (milled and roasted on dry heat)	Vanillic, syringic, melilotic, Cis ferulic acid
2.	Sukhdi (milled and roasted on dry heat)	Vanillic, syringic, p-OH benzoic acid
3.	Khichdi (soaked and cooked in open/closed pan)	Vanillic, syringic, p-OH benzoic acid, melilotic acid
4.	Kooler (milled and mixed raw with ghee and jaggery)	Vanillic, melilotic acid, p-coumaric acid
5.	Ghes (hand pounded, cooked with water and buttermilk)	Vanillic, syringic, p-OH benzoic acid, melilotic acid , p-coumaric acid

The results of present study (phenolic acid: p-hydroxy benzoic acid, vanillic, syringic, ferulic, p-coumaric) are similar to those brought back by *Dyke and Rooney* [51] in millets. The biosynthesis of phenolic compounds and organosulfur compounds is affected by different cultivation conditions, such as weather conditions, plant location, and harvest period [52].

Two classes of phenolic acids can be distinguished: derivatives of benzoic acid and derivatives of cinnamic acids. The hydroxyl benzoic acid content of edible plants is generally very low, with the exception of certain red fruits, black radishes, and onions. The hydroxyl cinnamic acids are more common than the hydroxyl benzoic acids and consist chiefly of p-coumaric, caffeic, ferulic acid, and sinapic acids. These acids are rarely found in the free form, except in processed food that has undergone freezing, sterilization, or fermentation. Phenolic compound ferulic acid has added health benefits as it battles cancer. Ferulic acid is the predominant bound phenolic form. The fact that ferulic acid could be identified in pearl millet emphasizes its potential role in the fight against cancer [53-58].

Table 9: The Color Reactions of Phenolic Acids

S. No.	Phenolic Acid	UV	Diazotized P-nitra Aline	Diazotized Sulphanic Acid
1.	P- hydroxy benzoic acid	-	Pink	Yellow
2.	Protocatechuic acid	-	Brownish- violet	Pink
3.	Vanillic acid	-	Purple	Orange
4.	Syringic acid	-	Blue	Red
5a.	Cis-o-coumaric acid	Bluish yellow	Purple	Orange
5b.	Trans-o-coumaric	Bluish yellow	Purple	Orange
6.	Melilotic acid	-	Purple	Orange-yellow
7a.	Cis-p-coumaric acid	Blue	Blue	Pink
7b.	Trans-p-coumaric acid	Blue	Blue	Pink
8.	Caffeic acid	Blue	Brown	Pink
9a.	Cis-ferulic acid	Blue	Bluish-green	Purple
9b.	Trans-ferulic acid	Blue	Bluish-green	Purple
10a.	Cis-sinapic acid	Green	Blue green	Pink
10b.	Trans-sinapic acid	Green	Blue green	Pink
11.	Chlorogenic acid	Blue	Blue-purple	Orange-yellow

Rapid advances in science and technology, increasing health care costs, changes in food laws affecting label and product claims, an ageing population, and rising interest in attaining wellness through diet are the major factors that are fueling interest in such foods, and several clinical trials have been conducted to confirm the relationship between the components in the diet and risk of disease or health condition [59-62]. Further studies are therefore planned to assess the prevalence of these problems in the population of pearl millet belts. Studies are also suggested to quantify these polyphenols, assess their antioxidant activity levels, and also assess their health benefits by conducting clinical trials.

Authors' contribution: VN and EB designed the study, NS and MD contributed in the laboratory analysis and interpretation. All authors contributed to the final manuscript. None of the authors had a conflict of interest. None of the authors has conflicting interest in this program.

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